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THE EFFECT OF VARIOUS DENATURANTS ON THE HAEMOGLOBINS OF ADULT AND CORD BLOOD

I. COMPARATIVE RATES OF DENATURATION[1]

F. D. WHITE AND AUDREY KERR

Abstract

At room temperature and pH 8.5, the effects of concentrated solutions of urea, nicotinamide, guanidine, sodium benzoate, and sodium salicylate upon the haemoglobins of adult and cord blood have been followed spectrophotometrically. In all cases the reaction curves were similar to those of alkali denaturation, but differed in the rates of reaction and failure to show any marked difference in rate between adult and cord blood. In no instance was the rate for cord blood greater than the corresponding rate for adult blood. The velocity constants for these experimental conditions have been calculated.

Except in the case of the nicotinamide reaction, absorption curves of the reaction products before and after reduction with sodium dithionite showed differences from the corresponding curves of alkali denaturation at pH 11.9. It is suggested this may indicate varying degrees of denaturation.

Introduction

When a dilute solution of sodium hydroxide is added to a haemolyzate of red cells, the bright red color is changed to brown, very rapidly if the cells are from human adult blood, but more gradually if fetal or cord blood has been used. It has been shown by Haurowitz (5) that this color change is due to denaturation and since it is accompanied by a change in the absorption spectrum, the course of the denaturation can be followed spectrophotometrically. Resistance to alkali denaturation is characteristic of fetal haemoglobin and has been used to determine the amount of this constituent in cord blood and in the blood of the newborn (1, 10, 14, 17). Haurowitz (6) has also shown that while the absorption spectra of haemoglobins from different animal species are not appreciably different from that of the human, wide variations exist in the rates of their denaturation by alkali.

Little attention has apparently been paid to the effect of denaturants other than sodium hydroxide upon adult and fetal haemoglobin, although Gardikas *et al.* (2) investigated the action of urea and sodium salicylate upon adult and fetal methaemoglobin and reported the latter to be denatured by urea at a faster rate than the former. This apparent reversal of the generally accepted "refractory" nature of the fetal pigment suggested the present study to us.

[1]Manuscript received November 30, 1956.

Contribution from the Department of Biochemistry, University of Manitoba, Winnipeg, Canada. This work was aided by a grant from the National Research Council of Canada.

The denaturing effects of urea, guanidine, nicotinamide, sodium salicylate, and sodium benzoate upon the haemoglobins of adult and cord blood have been investigated at room temperature (approximately 23° C.) and the rates of denaturation determined. All experiments were carried out at a pH of 8.5 in which the denatured protein remained in solution. Urethane was also investigated, but precipitation invariably occurred before denaturation was complete.

Experimental

General Procedure

In all cases fresh samples of oxalated blood (adult or cord) were centrifuged, the supernatant plasma removed, and the cells washed three to four times with 0.9% sodium chloride solution. Approximately 0.3 ml. of the washed cells were then added to 20 ml. of 0.01 *M* Sorensen's glycine–NaCl–NaOH buffer of pH 8.5, well shaken, and filtered. A 0.5 ml. quantity of this solution was transferred to a 10 mm. absorption cuvette containing 2.5 ml. of the same buffer solution, mixed, and the optical density read in either a Beckman DU or a Hilger Uvispek Spectrophotometer, at 578 mμ. The reading was then adjusted to an absorbancy of 0.7–0.9 by suitable dilution of the original solution, and this formed the control. The denaturing reagents were dissolved in buffer solution to give solutions with a final pH of 8.5, 2.5 ml. were mixed with 0.5 ml. of the haemoglobin solution in the cuvette, and spectrophotometric readings were taken immediately and at suitable time intervals which varied with the denaturant used. The reaction was considered to be complete when the final reading showed no appreciable change in 24 hours. At this point the contents of the cuvette should no longer show the absorption bands at 540 and 578 mμ, typical of oxyhaemoglobin.

Denaturants

In each case preliminary experiments were carried out to determine the concentration of denaturant which at the desired pH would have the greatest effect and still maintain the protein in solution. With urea, the most suitable concentration was found to be 50% and this, when mixed in the absorption cuvette with the haemoglobin solution, gave a final concentration of approximately 7 *M*. With nicotinamide, sodium benzoate, and sodium salicylate, solutions of 50%, 30%, and 20% respectively were used; the final concentrations being approximately 3.4 *M*, 1.7 *M*, and 1 *M*. To get a guanidine solution of pH 8.5, it was necessary to dissolve the hydrochloride in a much stronger and more alkaline buffer than in the previous cases. By utilizing a 0.1 *M* glycine–NaCl–NaOH buffer of pH 12.4, a 40% solution of guanidine-HCl was obtained at the required pH. This gave a final guanidine concentration of approximately 3.5 *M*. In this last case it was obviously impossible to use the haemoglobin–buffer solution as control owing to the difference in pH. Consequently the control value was taken as the reading of the haemoglobin–guanidine solution immediately after mixing, the rate of denaturation being sufficiently slow to render the error a negligible one.

Calculation

The calculation was carried out according to the method previously reported for sodium hydroxide denaturation (17), the percentage of undenatured haemoglobin (H) at time t being represented by the equation:

$$H = 100\ (D_t - D_0)/(D_a - D_0)$$

where D_a is the density reading of the control and therefore represented 100% undenatured haemoglobin; D_t is the reading at time t; D_0 the reading after complete denaturation. The curves were constructed by plotting the logarithmic values for H against time.

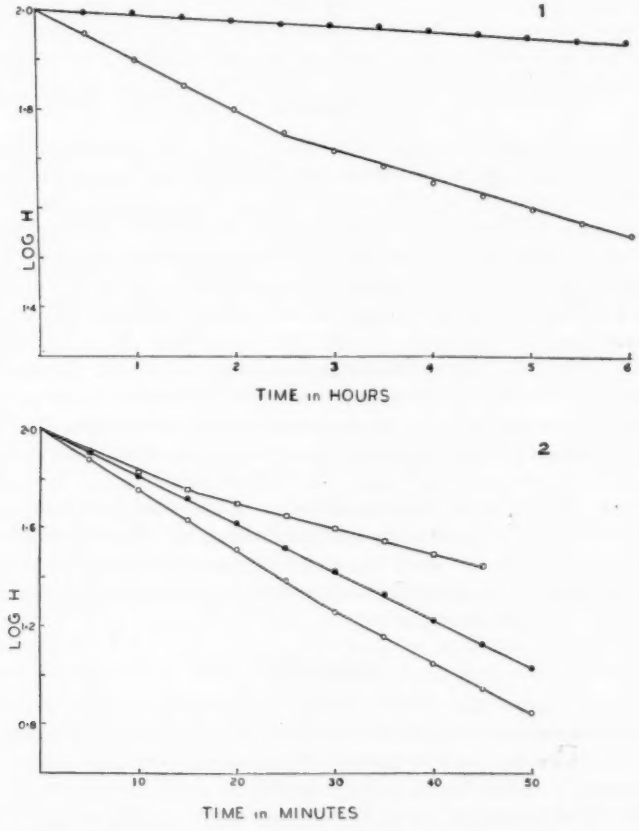

FIG. 1. Effect of urea upon adult and cord blood (H = percentage of undenatured haemoglobin). ○————○, adult; ●————●, cord.

FIG. 2. Effect of nicotinamide and guanidine upon adult and cord blood (H = percentage of undenatured haemoglobin). Nicotinamide: ○————○, adult; ●————●, cord. Guanidine: □————□, adult and cord.

TABLE I

RATES OF THE REACTIONS OF VARIOUS DENATURANTS WITH THE HAEMOGLOBINS OF ADULT AND CORD BLOOD

K = velocity constant: where reaction curve indicates two rates, K_1 represents initial velocity, and K, residual velocity

Denaturant	Adult Hb		Cord Hb	
	K (per sec.)	K_1 (per sec.)	K (per sec.)	K_1 (per sec.)
Urea	4.1×10^{-5}	6.6×10^{-5}	1.1×10^{-5}	—
Guanidine	3.8×10^{-4}	6.1×10^{-4}	3.8×10^{-4}	6.1×10^{-4}
Nicotinamide	7.9×10^{-4}	9.6×10^{-4}	7.4×10^{-4}	—
Sodium benzoate	2.5×10^{-3}	3.9×10^{-3}	1.9×10^{-3}	3.1×10^{-3}
Sodium salicylate	3.7×10^{-3}	6.4×10^{-3}	3.7×10^{-3}	5.3×10^{-3}
Sodium hydroxide (pH 11.9)	—	*5.7×10^{-2}	5.9×10^{-4}	—

*Since 95% of haemoglobin is denatured in 1 minute, the initial rate is the significant value.

Results

The results are shown in Figs. 1–3. In all cases the points represent mean values obtained from six to eight experiments carried out under the same conditions, the straight lines being drawn according to the method of least squares. Fig. 3 also shows typical curves of sodium hydroxide denaturation at pH 11.9, prepared for purposes of comparison (17). The comparative velocity constants calculated according to the formula: $K = [1/(t_2 - t_1)] \ln(H_{t_1}/H_{t_2})$ are shown in Table I. Where definite breaks in the curves indicated changes in the rate of reaction, the constant for the initial velocity (K_1) was calculated separately. The reaction rates showed a large variation, urea being the slowest acting, and sodium salicylate the fastest, although the latter rate was still markedly less than that for alkali denaturation of adult blood. With the exception of urea, very little difference was found in the reaction rates for adult and cord blood, and even with urea the difference was small in comparison with that for alkali denaturation (see Fig. 3). In no case, however, was there found any evidence of cord haemoglobin reacting at a faster rate than the adult form, and since the cord blood used generally contained about 20% of the adult component, the reaction rates for true fetal haemoglobin must be definitely less than the figures given.

Discussion

It has been shown that when strong concentrations of denaturants are added to haemoglobin solutions, a reaction occurs characterized by a progressive fall in the optical density measured at 578 mμ, similar to the decline observed when haemoglobin undergoes denaturation with sodium hydroxide. The respective rates of reaction, however, differ markedly with the reagent, and moreover do not show the pronounced difference between adult and cord haemoglobins characteristic of alkali denaturation. The question then arises: can these reactions be considered "denaturations" in the same sense of the term ?

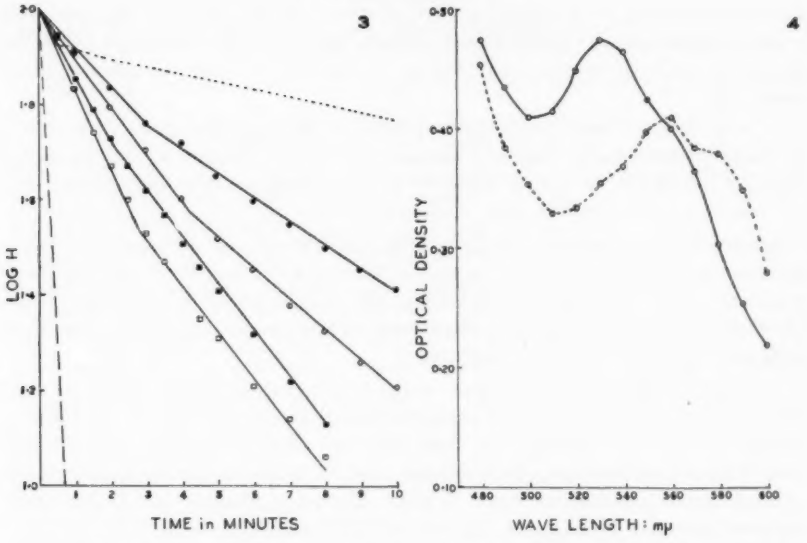

FIG. 3. Effects of sodium benzoate and sodium salicylate upon adult and cord blood, with curves of alkali denaturation at pH 11.9 for comparison. (H = percentage of undenatured haemoglobin). Sodium benzoate: O————O, adult; ●————●, cord. Sodium salicylate: □————□, adult; ■————■, cord. Alkali denaturation:—·—·—, adult; ....., cord.

FIG. 4. Typical absorption curves of urea denaturation product before (O————O) and after (O.....O) reduction with sodium dithionite.

When oxyhaemoglobin is denatured by sodium hydroxide the absorption bands at 578 and 540 mμ disappear and the product gives an absorption curve similar to, but not identical with, that of alkaline haematin (8). When this product, which we have referred to as alkaline globin haematin (17), is reduced by sodium dithionite ($Na_2S_2O_4$) it is converted to a haemochrome (the conjugation of ferroporphyrin with base (9)), with characteristic bands at 530 and 560 mμ. This denatured globin haemochrome can react to give stable haemochromes with basic substances, and in fact the pyridine and nicotinamide derivatives have been used as a means of determining the total haem pigments in blood (11, 12, 16). In the experiments now reported the products of the nicotinamide and guanidine reactions gave absorption curves similar to that of alkaline globin haematin, but whereas after addition of dithionite the nicotinamide product gave a typical haemochrome absorption curve, the guanidine curve showed only a plateau effect at 550–570 mμ. The absorption curve from the reaction with urea differed from the other two in showing a peak at 530 mμ somewhat similar to the spectrum which Haurowitz (4) ascribed to denatured globin haematin. This curve together with that of the corresponding reduced product is shown in Fig. 4. On addition of ammonia the guanidine and urea reduced products were both converted into typical haemochromes. The reactions with sodium benzoate and sodium salicylate

gave absorption curves similar to that of urea, but whereas on reduction with sodium dithionite a typical haemochrome spectrum was obtained with the benzoate, the salicylate precipitated and could not be examined spectrophotometrically.

It will be seen then that whereas only the nicotinamide reaction parallels spectrophotometrically that of alkali denaturation, the spectra of the other reaction products, modified possibly by the reagents themselves, are not inconsistent with some degree of denaturation.

Steinhardt's contention that the effect of urea is not denaturation but a splitting of the haemoglobin molecule (15) does not apply here since his work was carried out with horse haemoglobin, and it has been shown by Gutter *et al.* (3) that human haemoglobin is not dissociated by concentrated urea solution, as is the case with horse haemoglobin.

In experiments with 4.4 *M* urea on the methaemoglobins of adult and cord blood, Gardikas *et al.* (2) found that the fetal haemoglobin was denatured at a faster rate than the adult form, and that the addition of sodium dithionite after 8 hours in the case of the former and 24 hours in the latter produced typical haemochrome spectra. Under the experimental conditions now reported we have been unable to confirm these findings. Fig. 1 and Table I show that the reaction with cord blood proceeded at an appreciably slower rate than that with adult blood. Moreover, the absorption curve after reduction with sodium dithionite (Fig. 4) is obviously not typical of a haemochrome and this was obtained after 24 hours' denaturation. Further work is in progress on the nature of the urea reaction.

It should be emphasized that the velocity rates shown in Table I are comparative values and apply only to the experimental conditions under which they were determined. It was evident that the rate increased with the concentration of the denaturant, and consequently high concentrations were used, the limiting factor being the solubility of the haemoglobin in the final solution. There is therefore disparity in the percentage compositions of the denaturating solutions. Breaks occurred in all the adult and some of the cord haemoglobin curves shown in Figs. 1 to 3, indicating a diminution in the velocity of the reaction. Where alkali denaturation is concerned such breaks are generally held to denote the presence of two or more different types of haemoglobin (6), but the occurrence of a break appears to depend upon the rate of the reaction as determined by either the sodium hydroxide concentration or the pH. Thus Israels (7) has shown that, whereas the proportion of fetal haemoglobin in cord blood can be determined by the use of $N/4$ sodium hydroxide with a final pH of 11.9, when $N/10$ alkali is used (final pH = 11.3) the denaturation curve shows no appreciable break, while Rossi-Fanelli *et al.* (13) reported the apparent presence of three components in adult oxyhaemoglobin as deduced from the denaturation curve at pH 12.8 as against two components at pH 12.0. The precise significance of the breaks in the denaturation curves of these other reagents therefore can not be established until the nature of the reactions has been more fully investigated.

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OBSERVATIONS ON THE PHYSIOLOGICAL HALF-LIFE OF SERUM PROTEINS IN THE COCKEREL AND THE LAYING PULLET[1]

W. E. VANSTONE, W. F. OLIVER, W. A. MAW, AND R. H. COMMON

Abstract

The serum proteins of two cockerels and two pullets were labelled by oral administration of S^{35}-L-methionine. The subsequent decline of specific activity of serum proteins indicated physiological half-lives of approximately 6.5 days for both serum albumin and serum globulin in the cockerels. One pullet continued in active lay and the half-life of its serum albumin was approximately 8.5 days; the decline of specific activity of the globulin fraction displayed a relatively steep initial phase; the succeeding decline gave a half-life of approximately 4.6 days; the half-life of the serum phosphoprotein fraction was probably considerably less. These results are discussed in terms of estimated replacement rates of the proteins. The second pullet stopped laying and the results with this bird are discussed separately. The average specific activity of yolk protein attained a maximum in the egg laid on the 6th day after labelling. The subsequent decline in average specific activity of yolk protein with time followed a logarithmic course.

Introduction

It has been possible to distinguish six well-defined fractions on filter paper electropherograms of sera of immature pullets or cockerels (7, 6).

These fractions have been designated by analogy with the proteins of mammalian sera as albumin, α_1-, α_2-, and α_3-globulins, β-globulin, and γ-globulin. Electropherograms of the sera of 14-day embryos showed only albumin, α_2- and β-globulin, together with a prealbumin fraction, but the full normal pattern was established by the 7th day after hatching (18).

A seventh fraction appears in the serum of the pullet as reproductive activity begins, and a similar fraction appears in the sera of immature pullets or of cockerels after the administration of estrogen (7). This new fraction is associated with much lipid. The new fraction ("PP") is largely, if not completely, phosphoprotein (2, 7), and the γ-globulin fraction is either increased or augmented by an additional fraction (8). The phosphoprotein fraction of serum is regarded as yolk phosphoprotein that has been synthesized in the liver and is being transferred to the ovary.

Peters and Anfinsen (12) measured the rate of serum albumin production by chicken liver slices *in vitro*. On the basis of the rate found (0.12 mg. albumin per g. liver per hour) they estimated a turnover time of 13.4 days for the circulating serum albumin of a 2.5 kg. chicken. This corresponds to a physiological half-life of 9.3 days. The terms physiological half-life ($t_{1/2}$), turnover time (T_t), and replacement rate (R) are used throughout the present paper in the senses in which they have been defined by Tarver (17).

McKinley *et al.* (8) made a preliminary study *in vivo* of the physiological half-life of these serum protein fractions by the technique of Niklas and

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Maurer (10). A crossbred pullet, aged 8 weeks, was maintained on a uniform daily food intake in an individual cage. Two millicuries of S^{35}-L-methionine was then injected through the skin and wall of the crop directly into the crop contents, by means of a hypodermic syringe, while the bird was consuming its morning feed. A 2 ml. blood sample was withdrawn from the jugular vein an hour later, and similar samples were withdrawn at 1, 2, 4, 8, and 12 days thereafter. Electropherograms of the sera were prepared. The total radioactivity of each protein band was plotted on semilog paper against time. The results displayed a general resemblance to those reported by Niklas and Maurer (10) for the rat, and indicated a physiological half-life of 4.5 to 6.0 days for serum albumin.

If a serum protein fraction were being utilized preferentially for yolk formation, then one might expect the fraction to exhibit a relatively higher replacement rate and shorter half-life in the laying bird than in the cockerel or non-layer. The present paper describes experiments designed to investigate this point.

Experimental

Experimental Birds

Two Rhode Island Red pullets were placed in individual cages when they began to lay in mid-July, at which time they were 22 weeks of age. The birds were provided with laying mash, crushed oyster shell, and grit ad libitum. Two Rhode Island Red cockerels which had been hatched 3 weeks later than the pullets, but which were otherwise similar, were placed in individual cages a week before the beginning of the experiment. The cockerels were managed and fed in the same way as were the pullets.

Experimental Materials and Method

Twenty-five millicuries of S^{35}-L-methionine (Lot No. SMEL 5507) was obtained from Schwarz Laboratories, Inc. This preparation contained, on July 18, 3.06 mc./ml. (520 μc./mg. of methionine). The total volume of solution was 8.5 ml. of pH 1.2.

Two experiments were made. Each experiment comprised observations on one pullet and one cockerel. For each experiment, 4.25 ml. of the radioactive methionine solution was adjusted to pH 3.8 with NH_4OH and HCl solutions. Three-fifths of this solution was administered orally to each of the pullets and two-fifths to each cockerel. Each pullet, therefore, received approximately 6 mc. labelled methionine and each cockerel approximately 4.5 mc.

The first experiment was begun on August 1, on which date the food cups were removed from the birds' cages at 8.00 p.m. On the morning of August 2, the following schedule was observed:

8.00 a.m. Feed returned.
9.15 a.m. One-third of the dose of labelled methionine was squirted by means of a hypodermic syringe down the throat of each bird.
9.45 a.m. Feed removed.
10.45 a.m. Feed returned.

11.30 a.m. One-third of the dose of labelled methionine was administered as before.
12.00 noon Feed removed.
1.15 p.m. Feed returned.
1.30 p.m. One-third of the dose of labelled methionine was administered as before.

The radioactive methionine was administered in this way in order that absorption of methionine from the food might coincide as far as practicable with absorption of the radioactive methionine. The experiments were not designed for study of changes in radioactivity of serum proteins during the labelling period, otherwise it would have been necessary to define the time of administration more closely. (It will be seen below, however, that this would probably be necessary for measurements of the half-life of serum phosphoproteins.) Blood samples, approximate volume 3 ml., were withdrawn from the wing veins at 1, 2, 3, 6, 8, 10, and 12 days after administration of the radioactive methionine.

The second experiment was begun on August 14 and was carried out in the same way as the first experiment. The eggs laid by the pullets subsequent to labelling were held for examination as described below.

Analytical Methods

Total serum protein was determined by the biuret reaction (19). The total radioactivity of the serum was measured as follows: 0.1 ml. of serum was mixed in a steel counting cup with 0.9 ml. of a solution containing 1.0% NaCl, 0.05% Na_2CO_3, and 0.05% $NaHCO_3$. The samples were dried *in vacuo* over P_2O_5 and then counted under an end-window Geiger–Mueller tube.

Zone electrophoresis was carried out as described elsewhere (6), except in so far as the papers were supported on a sheet of pebbled Plexiglass as described by Sehon *et al.* (14). The papers were run for 30 hours at 5° C. to ensure good separation. The electropherograms were air-dried and counted at $\frac{1}{4}$ in. intervals under an end-window counter fitted with a slit $\frac{1}{4}$ in. wide cut in a lead shield. The papers were next freed from lipid by boiling in ethanol – ethyl ether (3 : 1 v/v) that contained 5% trichloroacetic acid, and were then boiled for 1 hour in each of three successive changes of ethanol–chloroform (1 : 1 v/v) that contained 5% trichloroacetic acid. The extracted papers were stained by the method of Kawerau (4), including the use of purified napthalene black. The stained papers were scanned with a Photovolt Densitometer Model 525 without filter. The areas under the peaks of the curves for radioactivity and for density of staining were used to calculate the activities and the amounts of protein in each fraction (18). Specific activities (ct./min./mg. protein) were calculated from these results.

The curves for radioactivity as measured before extraction of lipids from the papers were identical with those obtained after extraction of the lipids. This confirmed McKinley's observation (5) that the lipid extraction did not dislodge protein material from the papers.

TABLE I

LEVELS OF TOTAL PROTEINS AND OF CERTAIN ZONE ELECTROPHORETIC FRACTIONS OF SERUM PROTEINS IN THE EXPERIMENTAL BIRDS. ALL VALUES GIVEN AS G./100 ML.

Days since dosing with S^{35}-L-methionine	Cockerel No. 1			Cockerel No. 2			Pullet No. 1*				Pullet No. 2†			
	Total	Albumin	Globulins, $\alpha + \beta$	Total	Albumin	Globulins, $\alpha + \beta$	Total	Albumin	Globulins, $\alpha + \beta$	PP fraction	Total	Albumin	Globulins, $\alpha + \beta$	PP fraction
1	3.6	1.2	1.3	3.6	1.4	1.3	4.6	1.5	1.9	0.54	3.8	1.4	1.4	0.29
2	3.5	1.1	1.5	3.3	1.3	1.2	4.0	1.2	2.1	0.25	4.2	1.4	1.7	0.34
4	3.9	1.5	1.4	3.6	1.4	1.2	4.0	1.4	1.8	0.23	5.1	1.9	1.9	0.56
6	—	—	—	3.7	1.4	1.4	3.9	1.3	1.8	0.25	4.8	1.9	1.8	0.20
8	3.3	1.2	1.0	—	—	—	4.2	1.3	2.2	0.20	4.1	1.6	1.5	—
10	3.3	1.1	1.2	3.6	1.4	1.3	—	—	—	—	3.8	1.5	1.4	—
12	3.4	1.5	1.2	—	—	—	4.0	1.1	2.2	0.27	—	—	—	—
13	—	—	—	3.5	1.2	1.4	—	—	—	—	3.8	1.5	1.4	—

*In full lay throughout.
†In full lay before experimental period but laid last egg on day 1.

Eggs were broken and the yolks were separated by snipping off the chalazae with scissors and draining off most of the egg white. The yolk was then rinsed quickly in 1% NaCl and rolled on filter paper to remove adherent albumen and liquid. The vitelline membrane was slit and the content were collected and suspended in 2.5 volumes of 1% NaCl. This suspension was centrifuged and the supernatant was decanted and analyzed for radioactivity and protein fractions as described above for serum. The precipitate was washed twice with distilled water and was then dissolved in 10% NaCl, and the solution similarly analyzed for radioactivity and protein fractions.

Experimental Results

Cockerels No. 1 and No. 2

The experimental results for the two cockerels were in close agreement. The values for total serum protein, serum albumin, and serum α- plus β-globulins are presented in Table I and the specific activities of the albumin and total globulin fractions are plotted on a semilogarithmic scale against time in Fig. 1. The levels of total serum protein and of the six electrophoretic fractions studied were in general agreement with those determined previously by the same methods for cockerels of the same age (18). The specific activity of the albumin fraction (see Fig. 1) declined logarithmically after the 2nd day, and afforded an estimate of 6.5 days for the physiological half-life ($t_{1/2}$) of this fraction. This estimate, and the other estimates of $t_{1/2}$ discussed in this paper, are subject to the errors involved in the various assumptions on which they are based (17).

The corresponding curves for total serum globulin (see Fig. 1) displayed an initial steeper decline but this was almost over by the 2nd day. Thereafter the specific activity declined logarithmically. Niklas and Maurer (10)

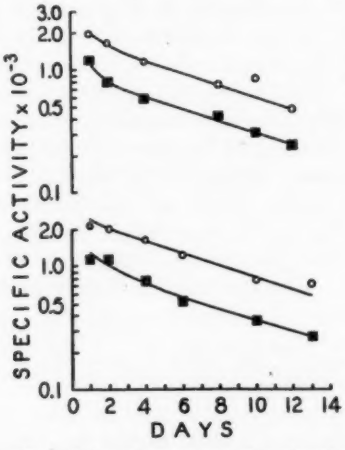

FIG. 1. Decline of specific activity of serum proteins of cockerels after administration of S^{35}-L-methionine. Upper pair of curves: cockerel No. 1. Lower pair of curves: cockerel No. 2. Circles: serum albumin. Squares: total serum globulin.

observed a similar steeper decline in the rat, and McKinley *et al.* (8) noted the same thing in the immature pullet. The difference between the albumin and the globulin fractions in this respect has been ascribed to relatively slower mixing of endovascular globulin with extravascular globulin (10). The later linear portion of the curves for total globulin afforded an estimate of 6.5 days for the half-life of total serum globulin. Similar examinations of the complete data suggested that the half-lives of α-, β-, and γ-globulin fractions did not differ greatly from this value.

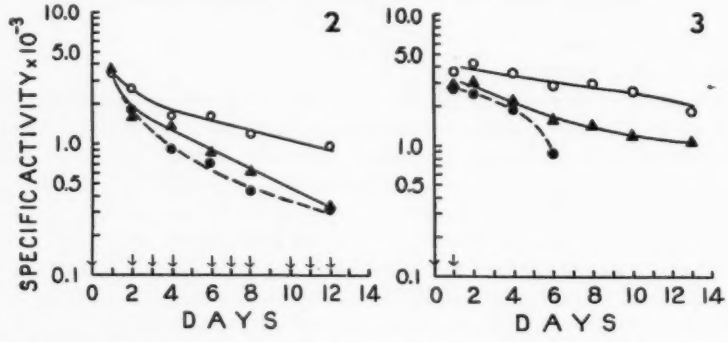

FIG. 2. Decline of specific activity of serum proteins of pullet No. 1 after oral administration of S^{35}-L-methionine. Circles: serum albumin. Triangles: α- plus β-globulins. Closed circles: serum phosphoprotein ("PP") fraction. Arrows: days of oviposition.

FIG. 3. Decline of specific activity of serum proteins of pullet No. 2 after oral administration of S^{35}-L-methionine. Circles: serum albumin. Triangles: α- plus β-globulins. Closed circles: serum phosphoprotein ("PP") fraction. Arrows: days of oviposition.

The values for total serum protein, serum albumin, α- plus β-globulins, and the phosphoprotein (PP) fraction for both pullets are presented in Table I. The specific activity of the serum albumin, the α- plus β-globulins, and the phosphoprotein ("PP") fraction are plotted on a semilogarithmic scale against time in Figs. 2 and 3. The designations used for the protein fractions of the serum of the pullets have been fully explained elsewhere (18). Both birds were still in full lay when placed in the experimental cages. Pullet No. 1 continued in lay thereafter. Pullet No. 2, however, went out of lay at the beginning of the experimental period and laid her last egg on the day after administration of the radioactive methionine. It will be seen below that the results for the two pullets differed as between each other as well as from the results for the cockerels. It seems very likely that the differences between the results for the two pullets is attributable to this fact, that one was in full lay throughout, whereas the other was passing into the midseasonal break in lay. For this reason the results for the two pullets are considered separately.

Pullet No. 1

The total serum protein was slightly higher than that of the cockerels and remained remarkably steady after the 1st day. This steadiness of the total protein level was in accord with the steady rate of egg production. There is

some evidence that serum protein may lie slightly higher in pullets than in cockerels at some stages of the laying cycle, though in full reproductive activity such differences may no longer be evident (18).

The specific activity of the serum albumin (see Fig. 2) displayed an initial curvilinear fall, but from the 4th day the decline was logarithmic and afforded an estimate for $t_{1/2}$ of 8.5 days.

The curve for α- plus β-globulins followed a similar general course, but the logarithmic part of the curve gave a value of $t_{1/2} = 4.6$ days. This contrasts with the results for the cockerels, where $t_{1/2}$ for the globulins was about the same as for the albumin.

In Fig. 2 the curve for the phosphoprotein ("PP") fraction is plotted separately. It displays a steep initial fall and also remains curvilinear fhroughout. This would be consistent with a replacement rate considerably in excess of that of serum albumin and serum globulins, with the logarithmic phase of decline of specific activity having been passed in the earlier stages of the experiment. Accordingly, no attempt has been made to arrive at an estimate of $t_{1/2}$ for this fraction from the curves.

Pullet No. 2

The total serum protein of pullet No. 2 rose to the relatively high value of 5.1 g./100 ml. on the 4th day after labelling, which was the 3rd day after the bird laid her last egg. Repeated bleeding would have tended, if anything, to lower serum protein level by hemodilution (16). The phosphoprotein fraction also rose to a maximum (0.56 g./100 ml.) on the 4th day, but thereafter it declined sharply and this fraction was no longer distinguishable on the electropherograms from pullet No. 2 after the 6th day. Consequently, no attempt was made to fractionate the γ-globulin region of the last three samples. The increase of serum protein level on cessation of laying may have been related to hemoconcentration at this phase of the reproductive cycle, because the increase affected all the electrophoretic fractions studied.

The slope of the curves for specific activity (see Fig. 3) indicated a value of approximately 12 days for $t_{1/2}$ for serum albumin and of 8 days for serum α- plus β-globulins, but these estimates are invalidated because of the sharp increase of serum protein level that followed the cessation of egg laying. The curve for the phosphoprotein ("PP") fraction fell very steeply so far as phosphoprotein could be measured separately. The rapid decline of phosphoprotein noted in the serum of pullet No. 2 on cessation of laying is in accord with the observations of Hosoda *et al.* (3).

Rates of Synthesis of Serum Proteins

The primary object of the present work was to compare the replacement rates of serum protein fractions in laying birds and cockerels. Calculations of replacement rates necessitated an estimation of pool size (P) in the different experimental birds.

The volume of serum, which is practically the same as the volume of plasma, was calculated from the average live weights by means of the regression

equations and average hematocrit values given by Newell and Schaffner (9). The average serum albumin values for all determinations on the cockerels and on pullet No. 1 were then used to estimate the total amounts of serum albumin in the blood as given in Table II. For purposes of further calculation these amounts have been taken as first approximations to the serum albumin pools (P). It is obvious that this is an underestimation. The serum albumin in the blood is of the order of 50% of the total exchangeable serum albumin in mammals (17) and, while no actual estimations for birds have been seen by us, the same kind of proportion probably applies also to birds. In any case, the necessary correction of the R values in Table II is easily made by multiplying by 2 on the assumption that 50% of the total exchangeable albumin is in the blood.

TABLE II

ESTIMATED REPLACEMENT RATES OF SERUM ALBUMIN IN THE FOWL

	Cockerels (av. of two birds)	Pullet No. 1 (in full lay throughout)
Live wt., g.	2630	2100
Calculated blood vol., ml.	251	157
Assumed blood cell vol., %	40	30
Estimated vol., serum, ml.	151	110
Average serum albumin, mg./100 ml.	1305	1280
Estimated serum albumin in blood vascular system, mg.	1965	1410
$t_1/_2$ (observed), days	6.5	8.5
Turnover time T_t, days	9.36	12.24
Replacement rate, R_1, mg./day	210	115
Replacement rate, R_2, assuming 50% exchangeable serum albumin in blood, mg./day	420	230
Synthesis of serum albumin by liver, mg./g. liver/hr. (based on R_2 above)	0.370	0.252

If it is assumed (17) that liver weights are 1.8% of the live weights, and that serum albumin is formed entirely, or almost entirely, in the liver, then it is possible to calculate rates of synthesis of serum albumin by the liver. The calculated values are 0.185 mg./g. liver/hr. for the cockerels and 0.126 mg./g. liver/hr. for the pullets, or double these values if blood serum albumin is assumed to be 50% of total exchangeable serum albumin as shown in Table II.

It must be remembered that these calculated values, as well as those in Table III, are based on a number of approximations and assumptions, all of which are subject to considerable error (17). This circumstance impairs their value as absolute measurements of replacement rates and rates of synthesis. Nevertheless, it seems reasonable to regard these estimates as providing useful indications of differences in rates of synthesis of various fractions. Furthermore, the estimates for rates of synthesis of serum albumin based on the present experiments are of the same general order of magnitude as those reported by Peters and Anfinsen (12). The latter workers found that chicken liver slices would produce 0.12 mg. serum albumin/g./liver/hr. *in vitro*. Our

TABLE III

COMPARISON OF ESTIMATED REPLACEMENT RATES OF SERUM ALBUMIN AND GLOBULINS IN THE FOWL

	Turnover time, T_t, days	Total serum content, mg.	Replacement rates, mg./day	
			R_1	R_2
Cockerels				
Albumin	9.36	1965	210	420
α- + β-Globulins	9.36	1925	206	412
Pullet No. 1				
Albumin	12.24	1410	115	230
α- + β-Globulins	6.63	2189	330	660

estimates based on total exchangeable serum albumin are roughly two to three times the value given by Peters and Anfinsen, but one might expect higher synthetic efficiency from liver *in vivo* than *in vitro*.

The presence of phosphoprotein, and probably of another yolk protein fraction (8), in the sera of the pullets introduced a complication into the estimation of replacement rates for serum globulins. However, the slopes of the curves for different globulin fractions (apart from the phosphoprotein) did not differ greatly. Accordingly, half-lives and replacement rates were estimated from the summation of the values for α- and β-globulins in the same way as described in Table II and the resultant estimates are included in Table III.

Table III shows that the replacement rates of serum albumin and serum globulin were nearly the same in the cockerels, whereas in pullet No. 1 the replacement rate of the α- + β-globulin fraction was nearly three times that of the albumin. This observation may be interpreted as evidence that the α- plus β-globulin fraction is utilized in egg production far more extensively than the albumin fraction.

A logarithmic phase of the decline of specific activity of the phosphoprotein fraction was not observed, and it was suggested above that this might be due to that phase having been passed. This is made more probable by the following considerations. Pullet No. 1 was laying at a rate not less than 50% (eggs laid ÷ no. days). The average vitellin content of an egg is 2.4 g. (13), so that this pullet was synthesizing at least 1.2 g. of phosphoprotein per day. If this was being synthesized in the liver and transferred to the ovary via the serum, and if the serum phosphoprotein is taken at its average value of 290 mg./100 ml., then the total vascular serum phosphoprotein was 319 mg. This gives a turnover time of 0.266 days and a theoretical estimate of 0.185 days for $t_{1/2}$. If the total exchangeable phosphoprotein is taken as five times as great, this still gives a value for $t_{1/2}$ of less than 1 day. It seems likely, therefore, that the value of $t_{1/2}$ for serum phosphoprotein is so short that the experiments now reported were unsuitable for its measurement because the labelling period itself was of the same order of magnitude as $t_{1/2}$.

Specific Activity of Egg Yolk Fractions

The specific activities of the phosphoprotein and the 'supernatant' fractions of the yolk proteins prepared as described above are plotted on semilog co-ordinates in Fig. 4.

The yolks of the eggs laid on the days of labelling did not contain radioactivity measurable by the techniques used. The second and last egg laid by pullet No. 2, on the day following labelling, contained negligible radioactivity. The second egg laid by pullet No. 1, 2 days after labelling, was lost by breakage; the specific activities of yolks of succeeding eggs laid by this bird increased to a maximum for the fifth egg, which was laid on the 6th day after labelling.

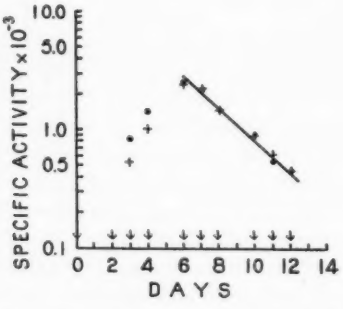

FIG. 4. Average specific activity of yolk protein fractions of eggs laid by pullet No. 1. Dots: phosphoprotein ("PP") fraction. Crosses: proteins of supernatant after precipitation of "PP" fraction. Arrows: days of oviposition.

NOTE: Yolk of eggs laid on day 0 did not contain radioactivity measurable by technique used. Egg laid on day 2 was broken.

Chargaff (1) administered disodium phosphate labelled with P^{32} to two laying hens by the intramuscular route. The specific activities of the P of yolk vitellin, lecithin, and cephalin attained maxima in the fifth egg laid by one of his birds after injection, and in the third egg laid by his other bird. These eggs were laid at 130 and 120 hours, respectively, after the injections. O'Neill *et al.* (11) found maximal recovery of P^{32} in the yolk of the fourth egg laid subsequent to a single oral dose of radioactive calcium phosphate. Spinks *et al.* (15) studied the appearance of P^{32} in hens' eggs after the oral administration of this isotope in the form of sodium glycerophosphate or sodium phosphate. Maximal uptake of P^{32} was observed in the fourth or fifth egg laid subsequent to dosage. The present results for oral administration of S^{35}-L-methionine agree closely with these previous results for the recovery of P^{32} in egg yolks after its administration to laying hens. In addition, the present results show that the decline in average specific activity of both of the yolk protein fractions that were examined followed closely similar logarithmic courses.

Acknowledgments

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THE ESTIMATION OF SUBSTITUTED THIOOXAZOLIDONES IN RAPESEED MEALS[1]

L. R. WETTER

Abstract

A quantitative method is described for the separate determination of thiooxazolidone and isothiocyanate in a single sample of rapeseed. The procedure involves first the estimation of the isothiocyanate, which is removed from the macerated meal by steam distillation. Then the filtered residue is shaken with diethyl ether and the quantity of thiooxazolidone in the extract is determined spectrophotometrically at 248 mμ. Recoveries of thiooxazolidone added to rapeseed meal are quantitative and precautions required to give reproducible results are outlined. The thiooxazolidone content of rapeseed meal and also the isothiocyanate/thiooxazolidone ratio vary considerably from sample to sample.

Introduction

Rapeseed meals are known to differ appreciably in their degree of toxicity under the same test conditions. These differences may be related to a variation in the content of two toxic components present in rapeseed meal—*l*-5-vinyl-2-thiooxazolidone (1, 6) and isothiocyanate (2, 7). Methods of analysis for these compounds were required for studies on the toxicity of rapeseed meals to animals and a satisfactory determination of isothiocyanates has been reported (7). A method for the estimation of thiooxazolidone has been described by Astwood (1). The present communication describes a modification of Astwood's procedure so that it can be used in conjunction with the estimation of isothiocyantes (7).

Hopkins (3) has reported that 5,5-dimethyl-2-thiooxazolidone is present in the seed of *Conringia orientalis*; therefore it is possible that thiooxazolidones, other than the *l*-5-vinyl derivative, are present in rapeseed. Since the procedure for estimation of substituted thiooxazolidones reported here is general for this class of compounds, the method therefore includes any thiooxazolidones which are released by enzymatic action.

Materials

Rapeseed meals were prepared in the laboratory by extracting ground rapeseed with Skellysolve "F".* Commercial samples of rapeseed meal were employed without further treatment.

Myrosinase was prepared from white mustard seeds according to a method described by Wrede (8). The final aqueous extract was freeze-dried and kept in the cold until ready for use. Three milliliters of a 0.5% (w/v) solution of

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*Petroleum ether (b.p. range 35°–58° C.) obtained from Skelly Oil Co., Kansas City, Mo.

myrosinase made up in 0.9% (w/v) NaCl were used for each assay. The diethyl ether employed for extraction purposes was redistilled after drying over metallic sodium.

The *dl*-5-vinyl-2-thiooxazolidone was synthesized by the method described by Raciszewski (6) and was a gift from Dr. L. W. Trevoy of the University of Saskatchewan.

Procedure

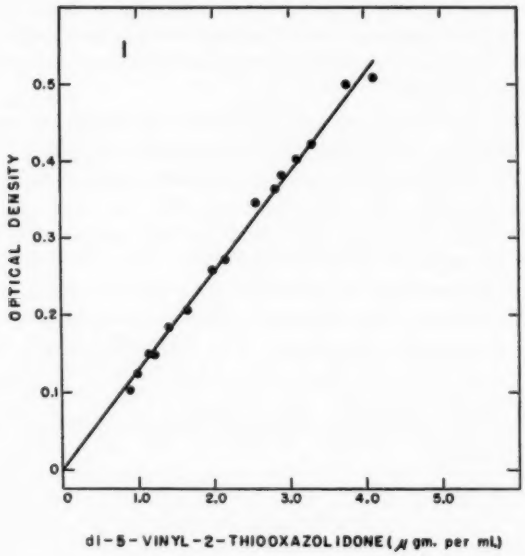
The meal was assayed for isothiocyanate as described in a previous publication (7), except that 3 ml. of myrosinase solution were used instead of white mustard powder. The importance of this change is discussed below. The residue suspension remaining from the isothiocyanate assay was filtered and the filtrate was used for the estimation of thiooxazolidone. The pH of an aliquot of the filtrate was adjusted to 10.5 with a known volume of 1 *N* NaOH, to prevent the subsequent extraction by diethyl ether of citrate, which absorbs strongly in the ultraviolet region. The neutralized solution (usually 1 ml.) was then extracted twice with 10 volumes of diethyl ether. The aqueous layer was discarded, and the absorption of the ether solution was measured at 230, 248, and 266 mμ with a DU Beckman spectrophotometer using diethyl ether as the blank.

The thiooxazolidone content was determined by comparing the corrected absorption at 248 mμ with a standard curve. The corrected absorption at 248 mμ was derived by subtracting the average optical density obtained at 230 and 266 mμ from that obtained at 248 mμ.

Results and Discussion

The calibration curve (Fig. 1) obtained with pure *dl*-5-vinyl-2-thiooxazolidone is linear over the concentration range employed in this study. The absorption spectrum for this material shifts towards the longer wave lengths when ether is employed as the solvent, while Astwood (1) found that in aqueous solutions the maximum absorption occurs at 240 mμ. The thiooxazolidone content in the rapeseed ether extracts was obtained by reference to the calibration curve and the total quantity in the original sample was calculated.

The commercial process for the recovery of oil from oilseeds generates enough heat to partially destroy the glucoside-splitting enzyme which is present in rapeseed. For this reason it is necessary to add enzyme to the commercial meal to obtain a quantitative estimation of thiooxazolidone. Early in the present investigation this was done by adding ground white mustard seed to the test sample. When the residue was neutralized with alkali and extracted with diethyl ether the spectrum shown in Fig. 2D was obtained. Since the amount of non-specific absorption is large it is not feasible to apply a correction as suggested by Astwood (1). However, a good estimation of the absorption of thiooxazolidone at 248 mμ can be obtained by employing either of the following methods. The correction method reported



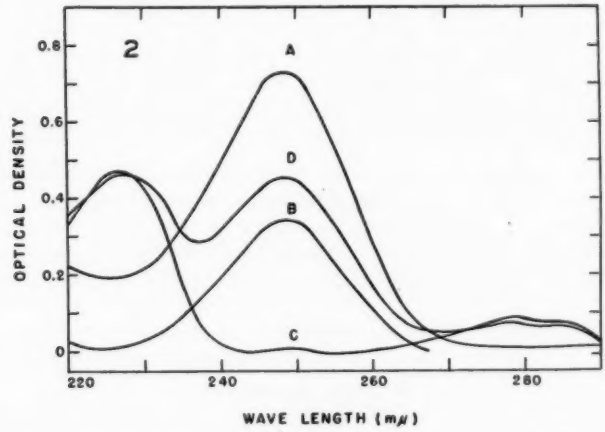


FIG. 1. The calibration curve obtained for *dl*-5-vinyl-2-thiooxazolidone when diethyl ether was used as the solvent. The values were obtained by carrying out the complete assay as described in the text.

FIG. 2. The ultraviolet absorption spectrum for various materials extracted by diethyl ether as described in the text. A: Polish rapeseed meal and myrosinase. B: Polish rapeseed meal alone. C: Mustard seed powder alone. D: Polish rapeseed meal and mustard seed powder.

by Kjaer *et al.* (4) was employed for the early part of this investigation by determining the optical density at 243, 248, and 253 mμ, and was successfully used to determine the thiooxazolidone content in samples when the non-specific absorption was high. A method described by Morton and Stubbs (5) also gave satisfactory results.

Meals prepared in the laboratory (see Materials), in which the enzyme had not been inactivated, gave absorption spectra practically devoid of non-specific absorption (Fig. 2B). When white mustard powder was added however, a spectrum showing considerable absorption at 227 mμ and 280 mμ was obtained (Fig. 2D), and a blank assay showed that the undesirable absorption was derived from the mustard powder itself (Fig. 2C). It is interesting to note that there is no appreciable thiooxazolidone present in mustard (Fig. 2C). Myrosinase prepared from white mustard powder was found to contribute very little to the non-specific absorption (Fig. 2A). Therefore, myrosinase was used in place of mustard powder in all subsequent assays. Experiments with three rapeseed varieties showed that they gave little, if any, non-specific absorption and therefore the simple correction method described was finally adopted (see Procedure).

The recovery of thiooxazolidone from rapeseed meals was investigated as follows: Known amounts of *dl*-5-vinyl-2-thiooxazolidone were added to Polish rapeseed meal. The mixture was macerated together with citrate buffer and myrosinase solution, and the isothiocyanate and thiooxazolidone contents were determined. A typical experiment (Table I) shows that the recoveries are good for the range studied and that the meal does not interfere with the recovery. The last column in Table I shows that the concentration of thiooxazolidone has no effect on the isothiocyanate assay in the same sample and that an estimation for both compounds can therefore be achieved.

The assay methods were utilized to determine the isothiocyanate and thiooxazolidone content of a few different samples of rapeseed (Table II). Wide variations in thiooxazolidone content of the meals are evident; an Argentine variety contained the highest amount while a Turkish variety tested contains none. There appears to be no correlation between the

TABLE I

THE RECOVERY OF VARYING AMOUNTS OF *dl*-5-VINYL-2-THIOOXAZOLIDONE FROM POLISH RAPESEED MEAL

dl-5-Vinyl-2-thiooxazolidone added to meal* (mg.)	Recovery of *dl*-5-vinyl-2-thiooxazolidone (%)	Natural isothiocyanate in meal (mg./g.)
0	0	5.79
0.91	105	5.94
1.81	97	5.87
2.72	99	5.77
3.62	100	6.01

*Two grams of rapeseed meal were used per test.

TABLE II

THE ASSAY OF RAPESEED MEALS FOR ISOTHIOCYANATE AND THIOOXAZOLIDONE

Meal	Mg. per g. of meal		Ratio*
	Isothiocyanate	Thiooxazolidone	
Argentine (1954)	4.06	6.80	0.60
Argentine (1955)	2.69	3.81	0.71
Argentine (commercial)	3.18	1.94	1.63
Polish (1954)	6.51	1.51	4.28
Polish (1955)	2.57	1.39	1.84
Polish (commercial)	2.65	1.67	1.58
Turkish	13.93	0.00	—

*Ratio $= \frac{\text{isothiocyanate}}{\text{thiooxazolidone}}$.

thiooxazolidone and isothiocyanate content. Thus the Turkish sample, which contains no thiooxazolidone, has the highest isothiocyanate content. The converse situation does not appear to exist; Argentine (1954), which had a high thiooxazolidone content, also had a considerable amount of isothiocyanate. The ratio given in the last column might suggest that varietal differences occur. A comparison of the 1954 and 1955 Polish samples, however, makes this appear unlikely but suggests that growing conditions could be important.

The assay outlined here together with the one described previously (7) should be useful in further studies regarding the toxicity of rapeseed meals to animals. Also these assays could aid plant breeders in selecting varieties where a knowledge of the total amount of these materials and their proportions are important.

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A STUDY OF STEROID COLOR REACTIONS IN STRONG ACID[1]

J. H. LINFORD

Abstract

Steroids react with acid reagents to produce compounds possessing intense absorption bands in the visible and ultraviolet spectral regions. These bands have been correlated with the existence of specific chemical groups in the steroid molecule. An attempt has been made to deduce the type of resonant system responsible for the absorption of light, in order to present a unified explanation of this type of steroid color reaction. The results indicate that the steroid nucleus, through induction or resonance effects, permits intercoupling between specific groups at the C_3, C_{11}, and C_{17} positions in a particular environment.

Introduction

The relatively small amounts of steroid materials obtained from natural sources gave early impetus to the development of physical methods for their recognition. At the present time the majority of these are based on the absorption of light. In the ultraviolet region this depends upon the presence of phenolic, keto, and conjugated keto groups; in their absence steroids are transparent. In the visible region the steroids do not absorb; however, the sensitivity of the eye to color, and later the convenience and sensitivity of radiant energy measurements in the visible region, produced a sustained interest in the formation of colored steroid complexes, which are complexes that absorb light in the visible region.

The steroid color reagents in common use are: (*a*) acids, with which dehydrating conditions may be present, as sulphuric, phosphoric, formic, trichloroacetic, and combinations of these with neutral solvents; (*b*) acids in conjunction with other reactive substances as furfural, formaldehyde, benzoyl peroxide, and acetic anhydride; (*c*) metallic salts in acid solutions, as antimony trichloride, arsenic trichloride, and zinc chloride; (*d*) certain chromogens as *m*-dinitrobenzene.

MacColl (21) and Dewar (7) have reviewed the attempts at the correlation of the color of an organic compound with its chemical constitution. Light absorption arises from the fact that a molecule can exist in any one of a number of different energy states. Chemically the higher energy states are represented by a polarization or separation of charge in the molecule. Molecules that absorb in the visible and near ultraviolet regions are generally capable of existing in mesomeric states, that is, they are hybrids of two or more classical structures of similar stability but different charge distribution; the molecule is considered to resonate between the various charged structures. Absorption of radiant energy brings about a displacement of the "equilibrium" between the several electronic states that contribute to the resonant state; the energy difference between the two lowest molecular states that contribute to the

[1]Manuscript received September 25, 1956.
Contribution from The Radiation Laboratories, The Manitoba Cancer Institute, Winnipeg, Man.

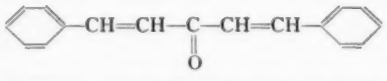
hybrid is equal to the energy corresponding with the longest wave length (lowest wave number) absorption band, providing the transition is permitted. The minimum excitation energy is less (wave number of absorption band is lower) for delocalized or π electrons than for those localized in definite atoms or bonds; mesomeric compounds contain these unsaturated linkages, which accounts for the correlation of mesomerism with color. The minimum excitation energy is also decreased by increasing the volume accessible to the unsaturation electrons, that is, by conjugation of groups containing delocalized electrons.

A resonating system, the absorption of which does not extend into the visible region, is termed a chromogen. Such a molecule contains one or more specific groups or atoms possessing this potentiality for color by reason of the presence of π electrons; this group is termed a chromophore. Auxochromes are groups capable of extending the resonance system of the chromogen to move the absorption into the visible. The distinction between chromophore and auxochrome is not absolute, as conjugated coupling of chromophores can produce color.

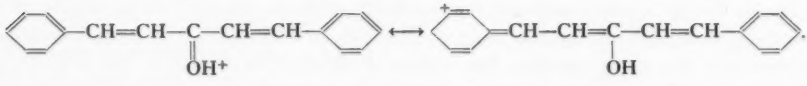
The reactions of organic compounds with mineral acids and metallic salts have been studied by Hammett (13), Newman (24), Gillespie (11, 12), and others. Practically all oxygen-containing compounds are soluble in sulphuric acid. Ethers and most carbonyl compounds behave as monoacid bases; they accept a proton from the solvent to form an oxonium salt, e.g.:

$$\rangle C{=}O + H_2SO_4 \longrightarrow \rangle COH^+ + HSO_4^-.$$

Ketones, especially α,β-unsaturated ketones, are apparently able to form more stable oxonium compounds than are the ethers. Aromatic unsaturated ketones of the type



form various colored compounds with sulphuric acid and with stannic chloride. Gillespie has shown that the color in acid is due to protonation of the carbonyl group. The resulting oxonium ion is intensely colored because of the possibility of resonance of a positive charge along a conjugated chain, e.g.:



The protonated cation forms a "halochromic" salt with the HSO_4^- ion.

Highly colored oxonium compounds are formed by the addition of electrophilic salts as $AlCl_3$, $SnCl_4$ to organic oxygen containing substances. These salts are non-electrolytes in the absence of water; they form molecular compounds as:

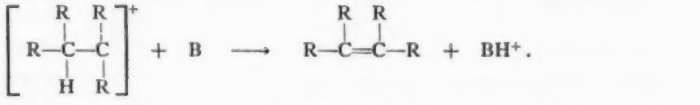
$$(C_6H_5)_2 . C{=}O + AlCl_3 \longrightarrow (C_6H_5)_2 . C{=}\overset{+}{O}{-}\bar{A}lCl_3 .$$

In sulphuric acid, primary aliphatic alcohols are converted to alkyl sulphuric acids. Secondary and tertiary alcohols give yellow colors and, if they are poured into ice water, unsaturated hydrocarbons may be obtained. In the latter instances complex ionization occurs; the oxonium ion first formed is not stable and undergoes further reaction with the solvent. Water is lost and a colored carbonium ion is formed, e.g.:

$$(C_6H_5)_3 . C{-}OH + H_2SO_4 \longrightarrow [(C_6H_5)_3 . COH_2]^+ + HSO_4^-,$$
$$[(C_6H_5)_3 . COH_2]^+ + H_2SO_4 \longrightarrow (C_6H_5)_3 . C^+ + H_3O^+ + HSO_4^-.$$

The freezing point depression of the sulphuric acid is four times that produced by a non-electrolyte and the solution is an intense yellow.

A carbonium ion may lose a proton to a base, to yield an olefin, e.g.:



Unsaturated systems also may react with strong acid to form a carbonium ion:

$$(CH_3)_2 . C{=}CH_2 + OH_3^+ \longrightarrow (CH_3)_3 . C^+ + H_2O.$$

The carbonium ion may, in turn, attack a double bond:

$$(CH_3)_3 . C^+ + (CH_3)_2 . C{=}CH_2 \longrightarrow (CH_3)_2 . C^+{-}CH_2{-}C . (CH_3)_3.$$

The polymerized carbonium ion may unite with the acid radical, or stabilize itself by attracting an electron pair from a C atom adjacent to the electron deficient C atom; this establishes a double bond involving the latter. The proton is expelled to the catalyst.

In the present paper, the absorption bands associated with a number of steroid color reactions have been compiled, and an attempt has been made to explain their occurrence on the basis of the above ideas. Connections of certain characteristic chemical groups in the steroids with specific absorption bands have been traced through a series of acid reactants. The latter are those more frequently referred to in the literature and are discussed in order of the increasing complexity of spectra.

Results and Discussion

The source of the steroids, and the methods of making the absorption measurements, were as described in earlier reports (17, 19, 20). The results are described and discussed under the heading of each solvent or reagent used to contain the absorbing compound. The spectral positions of the absorption bands are measured in wave numbers, on which scale the shape of the band represents more directly the absorption event. The intensity of absorption is listed as the optical density of the concentration named, for a 1.00 cm. path length; a series of dots indicates a broad general absorption over that region; the maxima not definitely resolved, that is, those that appear as "shoulders", are in parentheses.

TABLE I

THE SPECTRAL POSITIONS AND OPTICAL DENSITIES OF THE ABSORPTION MAXIMA OF STEROIDS DISSOLVED IN ABSOLUTE ETHYL ALCOHOL, CALCULATED TO CONCENTRATIONS OF 100 μG. PER MILLILITER, FOR 1.00 CM. THICKNESS

Steroid	Absorption maxima ($\times 10^{-2}$ cm.$^{-1}$)			Optical density	ϵ
Estradiol-17β		354		0.696	1,895
Estradiol-17α		354		0.744	2,027
Estrone		354		1.112	3,007
Testosterone			416	5.94	17,100
Progesterone			414	5.70	17,900
Desoxycorticosterone			415	4.86	16,000
Androsterone	340			0.0221	64
			413	0.0426	124
Epiandrosterone	345			0.0154	45
Dehydroepiandrosterone	345			0.0208	60
			410	0.0137	50
Ergosterol		340		1.65	6,500
		353		2.67	10,600
		368		2.52	10,000

NOTE: The associations of the absorption with the phenolic, conjugated keto, and keto groups are evident in the table.

Formic Acid

Representative phenolic, keto, and alcoholic steroids were dissolved in 90% formic acid at 80° C., for 10 minutes; absorption measurements at room temperature were made within the following ½ hour. Measurements on estradiol-17α and estradiol-17β were also made in 98% formic acid (see Table II).

In 90% formic acid, similarities in absorption exist within the estrogen series and within the series possessing the conjugated ketone group; the maxima were clearly resolved. No visible color was evident except a pale pink color with estradiol-17α (*trans*). The absorption of dehydroepiandrosterone, androsterone, epiandrosterone, cholesterol, and 3-cholestanol was negligible up to 42,000 cm.$^{-1}$, the limit of the measurements.

The use of 98% formic acid as a solvent produces a marked dissimilarity in the absorption of the two epimers of estradiol. The ultraviolet maximum of estradiol-17β increased; that of estradiol-17α decreased to 1/10th and maxima appeared in the visible region.

Using 98–100% formic acid Boscott (4) obtained no color with progesterone, androstanedione-3,17, and androstenedione-3,17. Colored products were obtained only in the presence of certain C_{17} hydroxyl groups and only in the presence of the C_{17} α-orientation in the cases of *cis* and *trans* testosterones and *cis* and *trans* estradiols. This color was attributed to the formation of a Δ^{16} double bond. Whether color was present or not, the steroids in the 98% acid solution showed fluorescence.

TABLE II

SPECTRAL POSITIONS AND OPTICAL DENSITIES OF THE ABSORPTION MAXIMA OF STEROIDS DISSOLVED IN FORMIC ACID AT 80° C. FOR 10 MINUTES

Concentration 100 μg. per ml. Cell thickness 1.00 cm.*

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-3}$)					
	388–385	363–360	325	240	225	190
90% formic acid						
Estrone		0.831	0.481			
Estriol		1.10	0.34			
Estradiol-17β		0.602				
Estradiol-17α		0.943				
Progesterone	2.79					
Testosterone	3.23					
Desoxycorticosterone	3.31					
98% formic acid						
Estradiol-17β		0.998			0.025	
Estradiol-17α		0.096		0.235		0.190

*Measurements made in 0.10 cm. cell (18).

Comparison of Tables I and II shows a change in the positions of the absorption maxima, brought about by the acid solvent. The shift to higher wave numbers of the absorption of the phenolic group is probably a solvent effect due to the change in the polar nature of the surrounding medium, but protonation of the phenolic group cannot be ruled out on the direction of the shift alone. The change in the absorption of estradiol-17α only, under dehydrating conditions, indicates that a new chromophore is formed at the C_{17} position. This may be the $\Delta^{16,17}$ double bond described by Boscott. The formic acid then acts as an auxochrome to produce a colored and fluorescing complex, probably a carbonium ion, but again, protonation at the -OH group is not ruled out. It is worthy of note that this reaction at the C_{17} position has markedly affected the intensity of absorption of the phenolic A ring.

In the 90% formic acid, the shift of the absorption of the conjugated keto groups to longer wave lengths (lower wave numbers), and the occurrence of fluorescence, which we have noted, indicates that a "color reaction" has occurred. Since this effect is the same for C_{17} keto and C_{17} -OH steroids, we assume that protonation of the C_3 keto oxygen conjugated with the double bond has occurred.

Phosphoric Acid

The absorption spectra of steroids in anhydrous phosphoric acid have been studied by Kalant (16), and by Nowaczynski and Steyermark (25). Finkelstein, Hestrin, and Koch (10) observed that estrogens dissolved in 85% phosphoric acid, heated for 30 minutes in a boiling water bath, formed

TABLE III

SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF 100 μG. OF STEROID IN 3.0 ML.* OF 85% PHOSPHORIC ACID HEATED 98° C. 30 MINUTES

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-3}$)					
	395	380	375	363	307	295
Estrone	0.39	0.37		0.39		
Estriol	.....	.....	.....	0.06	.....	.....
Estradiol-17β	.....	.....	.....	0.06	.....	.....
Estradiol-17α	.....	.....	.....	0.06	.....	.....
Progesterone		1.25	1.25			
Testosterone			1.16			
Desoxycorticosterone		1.27				
Dehydroepiandrosterone	.....	.....	.....	.....	(0.47	0.44)
Androsterone	No absorption					
Epiandrosterone	.....	.....	.....	0.22	.....	.....
Cholesterol	No absorption					

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-3}$)							
	262–254	246–242	240–237	220	210	200–195	180	168–165
Estrone				0.98			0.80	0.24
Estriol			1.14	.....		0.88		
Estradiol-17β				2.94		(0.74)		
Estradiol-17α				2.27		1.90		
Progesterone	No absorption							
Testosterone	0.18	(0.13)			0.16		0.06	0.07
Desoxycorticosterone		0.16			0.22			(0.06)
Dehydroepiandrosterone	0.33	0.23			.....	0.13		
Androsterone	0.46	0.47						
Epiandrosterone		0.28	0.27					
Cholesterol	No absorption							

*Concentrations are one-third those of Table II.

fluorescing solutions. The absorption maxima of a representative number of steroids treated in this way are shown in Table III.

The ultraviolet absorption of the estrogens in phosphoric acid resembles that in formic, although additional maxima have appeared, notably in the estrone solution; compare Tables II and III. The absorption at 36,300 $cm.^{-1}$ persists, that of estrone is of the same order of intensity but the estradiols and estriol have dropped to one-fifth in Table III. All four estrogens have absorption bands in the visible region; those of the estradiols and estriol are of the greatest intensity.

The nature of the C_{17} reaction may be conjectured if we accept the dehydration of estradiol-17α in 98% formic acid. Protonation of C_{17} hydroxyl and C_{17} keto permits the phenolic absorption in the ultraviolet at 36,300 $cm.^{-1}$, and causes visible absorption at 22,000 $cm.^{-1}$ and 16,500 to 20,000 $cm.^{-1}$. Carbonium ion formation, with loss of water, at the C_{17} hydroxyl group produces absorption at 22,000 to 24,000 $cm.^{-1}$ and markedly reduces the intensity at 36,300 $cm.^{-1}$. This indicates, as in the case with formic acid, that the C_{17} substituent has altered the reactivity of the phenolic group.

The visible region maxima of estrone must be due then to protonation of the C_{17} keto group. It is interesting to note that maxima at 22,000 $cm.^{-1}$ arise from a positive charge at the C_{17} position whether caused by protonation or carbonium ion formation; the former does not affect the C_3 absorption, the latter does. It is not surprising that the protonated ketone has an effect

similar to a carbonium ion, since the hybrid could resonate between the oxonium and carbonium ion structures, e.g.:

The additional maxima of estrone at 18,100 cm.$^{-1}$ and 17,000 cm.$^{-1}$ are evidently due to protonation of the keto group; weak bands in the same position are shown with testosterone and desoxycorticosterone. It is, therefore, probable that the estrogens in phosphoric acid exist both in the C_{17} protonated and C_{17} ionic forms; an equilibrium between these forms and the environment exists, and the maximum at 36,300 cm.$^{-1}$ is sensitive to a small degree of ionic character. The broad band of high intensity associated with estriol indicates a complex mixture of chromophores has been formed, probably of the ketonic type as well as unsaturated linkages.

The absorption at 37,500–38,000 cm.$^{-1}$ of the steroids possessing the conjugated keto group is similar in position and intensity to that shown by the same steroids in formic and trichloroacetic acid solutions and is associated with protonation of the C_3 ketone group. Visible absorption occurs with the keto steroids possessing a hydroxyl group; the bands in the 24,000–26,000 cm.$^{-1}$ region which are common to all arise from loss of the hydroxyl group and formation of a carbonium ion. Cholesterol is transparent, showing that interaction of the carbonium ion with the protonated keto group produces this absorbing system. The bands in the 21,000 cm.$^{-1}$ region occur only when a keto group is present in the sterol molecule together with the double bond and appear to be dependent on their relative positions; thus a double bond in the B ring of dehydroepiandrosterone shifts the band to 20,000 cm.$^{-1}$. The weak bands in the 16,500–18,000 cm.$^{-1}$ region, caused by protonation of the C_3 conjugated keto group, appear to require the presence of the carbonium ion at the C_{17} position, another instance of the relative dependence of these two atoms.

Some experiments were carried out using as the reagent 85% phosphoric acid to which had been added an amount of phosphorous pentoxide calculated to react with the 15% of water. The change in the reagent made no difference to the estradiols in the ultraviolet; in the visible region, estriol did not differ; the absorption of the estradiols and estrone at 20,000 cm.$^{-1}$ was greatly reduced leaving only one maximum, at 22,000 cm.$^{-1}$; the value of the 22,000 cm.$^{-1}$ maximum of estrone was increased threefold. The concentrated reagent made some difference to the dehydration reaction and subsequent carbonium ion formation at the C_{17} -OH groups, and made a marked increase in the degree of protonization of the C_{17} keto oxygen of estrone.

Trichloroacetic Acid

The formation of colored compounds by steroids and hydrocarbons in trichloroacetic acid solutions has been studied by Rosenheim (27), by

TABLE IV

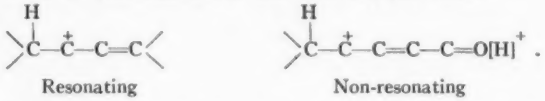
SPECTRAL POSITIONS AND OPTICAL DENSITIES OF THE ABSORPTION MAXIMA OF STEROIDS DISSOLVED IN THE TRICHLOROACETIC ACID REAGENT OF ROSENHEIM (27)

Concentrations 100 μg. per ml. Cell thickness 1.00 cm.

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-2}$)			
	370	360	175	160
Ergosterol	1.22		0.350	0.332
Cholestenol		0.08		
Testosterone	1.54			
Progesterone		1.35		

Schoenheimer and Evans (28), and by Miescher (22). The results obtained by these authors indicate that color reactions are undergone by compounds with conjugated double bonds, or with a hydroxyl group in the α,β position to a double bond (e.g. Δ^4-cholestenol). The latter is readily dehydrated to a conjugated double bond by Rosenheim's trichloroacetic acid reagent. However, conjugated keto groups, or even keto groups adjacent to conjugated double bonds (e.g. oxycholesterilene), did not form colored products.

Results obtained in this laboratory with Rosenheim's reagent are in agreement with these observations (see Table IV). Ergosterol developed a strong red color. Cholesterol showed practically no absorption. The conjugated keto steroids testosterone and progesterone formed no colored products, but absorbed strongly in the ultraviolet, in the same region as ergosterol. These results confirm the importance of the conjugated double bond system as a chromophore, and the inhibitory effect of the carbonyl group. The latter effect may be explained on the basis of destruction of the resonating system by protonation of the keto oxygen, thus:



Resonating Non-resonating

Protonation of the C_3 keto oxygens of testosterone and progesterone is indicated as in the case of the formic and phosphoric acid solvents.

Chlorides of Arsenic and Antimony

Kahlenberg (15) observed that cholesterol dissolved in arsenic trichloride at room temperature to form a red solution; at 0° C. color did not appear immediately, but colored solutions remained colored. Addition of benzene, toluene, or chloroform to the solution discharged the color; addition of water discharged the color and threw the cholesterol out of solution. Two grams of cholesterol were dissolved in 5 ml. of arsenic trichloride by boiling; the dark cherry red solution stood overnight at −10° C. when crystals of pure cholesterol separated out at nearly 100% recovery.

Cholesterol rubbed with antimony trichloride produced a complex initially pink in color that changed to brown; when dissolved in antimony pentachloride

a brown solution was formed; by using chloroform as a solvent for the pentachloride reagent, the solution formed was cobalt blue in color. An addition product of one molecule of cholesterol with one molecule of antimony pentachloride was found.

Pincus (26) used a concentrated solution of antimony trichloride in a nine to one glacial acetic acid – acetic anhydride solvent, as a color reagent for certain 17-ketosteroids. The reagent was added to the dry steroid and heated at 100° C. for 20 minutes. The cooled product was diluted with acetic acid, chloroform, or acetic anhydride. Androsterone and epiandrosterone developed an intense blue color; pregnanediol, cholesterol, testosterone, dehydroepiandrosterone showed slight absorption; the absorption of androstenedione, progesterone, cholestenone, and pregnanolone was negligible.

Absorption measurements carried out in this laboratory are shown in Table V. Those in the ultraviolet were made using steroid concentrations of 500 μg. per ml. in 0.10 cm. cells (18), owing to the high absorption of the reagent.

TABLE V

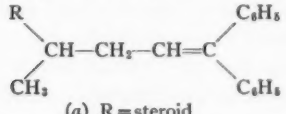
SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF 100 μG. STEROID IN 1.0 ML. PINCUS REAGENT INCLUDING ACETIC ACID DILUENT

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-2}$)				
	338	318	247	180	167–164
Dehydroepiandrosterone		0.43	0.61	0.942	1.20
Androsterone	1.33		1.18		3.22
Epiandrosterone		0.60	0.78		4.07
Testosterone	No absorption				
Progesterone	No absorption				

These results show that intense absorption, extending through the visible region into the ultraviolet, exists only for the C_{17}-keto steroids possessing an hydroxyl group at the C_3 position; this absorption is diminished by unsaturation at the C_5 position. Alcohols absorbed little, the conjugated C_3 keto group was transparent in contrast to the results obtained with formic and phosphoric acids.

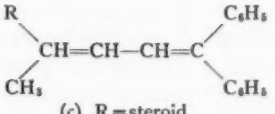
We note here again an interdependence of the C_3 -OH group and the C_{17} ketone group in the production of a resonating system having maximum absorption intensity in the lowest frequency band. The absorbing complex may be a molecular compound of the keto oxygen with the metal.

The compounds examined by Miescher (22) in trichloroacetic acid were also tested by him with antimony trichloride dissolved in chloroform. Of the four types of compounds examined:



(*a*) R = steroid
(*b*) R = —CH_3

and



(*c*) R = steroid
(*d*) R = —CH_3

(*b*) became yellow to green after 1 hour; (*d*) became red immediately; steroid derivatives (*a*) and (*c*) showed no reaction. The resonating system produced by reaction of the electrophilic salts with the double bond is apparently neutralized by the steroid reaction.

Concentrated Sulphuric Acid

Wokes (32) found that cholesterol, cholestene, and cholesterilene ($\Delta^{3,5}$ cholestadiene) produced similar red colors when dissolved in sulphuric acid, or arsenic or antimony trichloride. Axelrod (2) used the absorption spectra of steroids in both concentrated and fuming sulphuric acids to identify fractions on paper chromatograms. Zaffaroni (33, 34) has measured the absorption of a series of corticosterone derivatives in concentrated sulphuric acid C.P. at room temperature within the range of 16,500 cm.$^{-1}$ to 45,500 cm.$^{-1}$ (see Fig. 1 and Table VI) taken from his published figures and tables.

Androstane-3,11,17-trione, pregnanedione, and dehydrocholic acid show negligible absorption throughout this region. A maximum in the region of 35,000 cm.$^{-1}$ (285 mμ) persists throughout the series of Table VI except when C_3 (β) hydroxyl is present, as in compounds XIII, XIV, XV, and XVII; note column 1.

Provided that the 35,000 cm.$^{-1}$ maximum is present, a pair of absorption bands in the regions 28,200–30,000 cm.$^{-1}$ (333–355 mμ), column 3, and 24,000–24,400 cm.$^{-1}$ (410–415 mμ), column 5, are associated with the presence of the C_{11} keto group, independently of the C_3 group being keto or α-hydroxy, or the presence of unsaturation; C_{17} carboxyl displaces these bands, compounds XVII and XVIII. Provided that the 35,000 cm.$^{-1}$ maximum is present, replacement of the C_{11} keto by C_{11} hydroxyl or $\Delta^{9,(11)}$ double bond results in absorption at 25,500–27,000 cm.$^{-1}$ (373–390 mμ), column 4; compare compounds I, IV, and XV with compounds containing C_{11} keto, and hydroxy groups, other than 3 (β). In the absence of the 35,000 cm.$^{-1}$ absorption band, a pair of absorption bands in the regions 24,000–24,400 cm.$^{-1}$ (410–415 mμ), column 5, and 30,000–31,700 cm.$^{-1}$ (315–333 mμ), column 2, indicates a C_3 (β) hydroxyl, compare compounds XIII, XIV, XV and XVII.

The relationships noted above have occurred in C_{21} compounds possessing a C_{20} keto group and having various combinations of keto and hydroxyl groups at the C_3, C_{11}, and C_{17} positions, and in the C_{17} side chain. C_{20} carboxyl reduces the 24,000 cm.$^{-1}$ (415 mμ) band to the 24,700 cm.$^{-1}$ (405 mμ) positions; a 3,9 epoxy bridge acts as a conjugated C_3 keto group or an (α) hydroxy group in permitting the 35,000 cm.$^{-1}$ (285 mμ) absorption, since carboxyl in the presence of keto groups only is transparent, e.g. dehydrocholic acid, compare compounds XVII and XVIII.

In this series of compounds, absorption extends to the visible region, columns 5, 6, and 7, as an hydroxyl group is present at one of the C_3, C_{11}, C_{17}, or C_{21} positions. The removal of the C_{11} keto group, or its replacement by hydroxyl, results in visible absorption at longer wave lengths, compare compounds I, II, and III, compounds IV and V, compounds VII, VIII, and X.

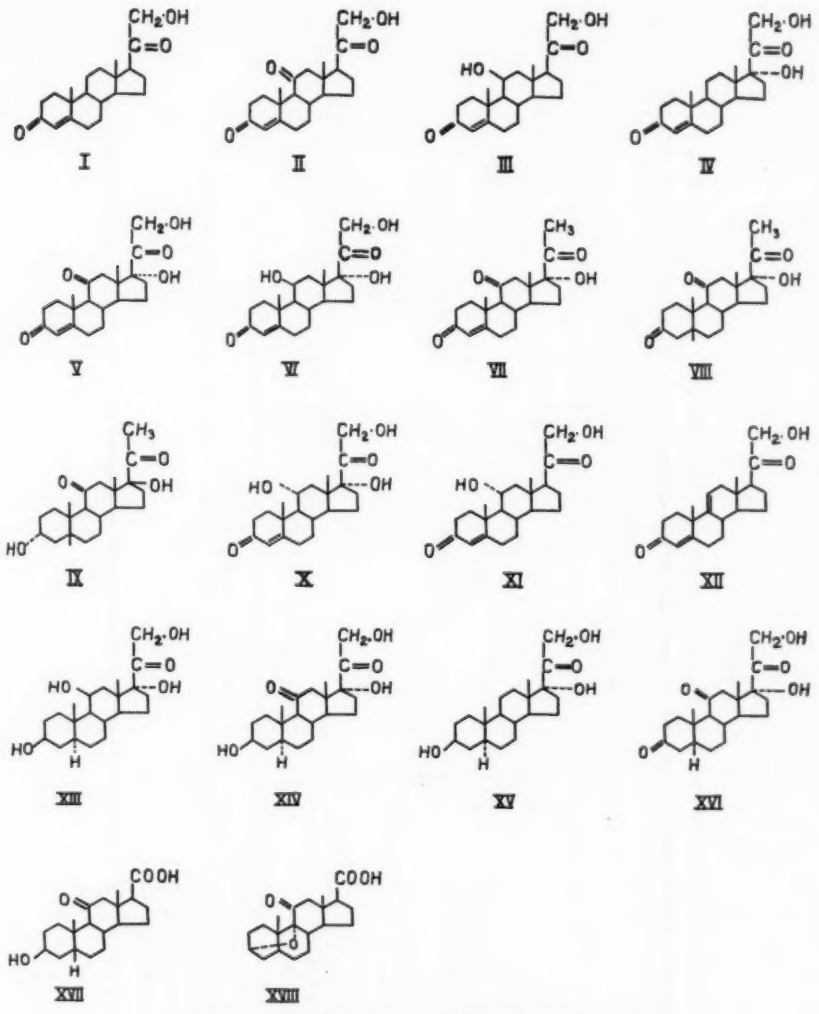


FIG. 1. Formulas of compounds listed in Table VI.

The presence of C_{17} hydroxyl in the absence of C_{11} keto or double bond produces absorption at longest wave lengths, column 7, compounds IV, X, and XIII.

The above tabulation indicates that absorption is associated with specific keto and hydroxyl groups. A dependence on the configuration of the hydroxyl group is evident; we may assume this controls the ease of formation of a double bond and hence a carbonium ion, the β compounds undergoing dehydration less readily. The absorption due to the C_{11} group is different for

TABLE VI

SPECTRAL POSITIONS OF THE ABSORPTION MAXIMA OF CORTICOSTERONE DERIVATIVES IN SULPHURIC ACID C.P.

	Steroid	Wave numbers ($cm.^{-1} \times 10^{-2}$)						
		(1)	(2)	(3)	(4)	(5)	(6)	(7)
I	11-Desoxycorticosterone	351			270		227	
II	11-Dehydrocorticosterone	357		282		241		
III	Corticosterone	351	303		268		220	
IV	11-Desoxy-17-hydroxycorticosterone	339						187
V	11-Dehydro-17-hydroxycorticosterone	353		294		241	210	
VI	17-Hydroxycorticosterone	357			256		210	
VII	Δ^4-Pregnene-17α-ol-3,11,20-trione	351		286		241		
VIII	Pregnane-17α-ol-3,11,20-trione	357		286		244		
IX	Pregnane-3α,17β-diol-11,20-dione	357		286		241	(222)	
X	Δ^4-Pregnene-11α,17α,21-triol-3,20-dione	351			256		213	185
XI	Δ^4-Pregnene-11α,21-diol-3,20-dione	351			256		213	
XII	$\Delta^{4,9(11)}$-Pregnene-21-ol-3,20-done	351	303		270		217	
XIII	Allopregnane-3β,11β,17α,21-tetrol-20-one		303			241		196
XIV	Allopregnane-3β,17α,21-triol-11,20-dione		300			244		
XV	Allopregnane-3β,17α,21-triol-20-one		317			244		
XVI	Pregnane-17α,21-diol-3,11,20-trione	370		294		241		
XVII	3-Hydroxy-11-keto-etiocholanic acid		312			247		
XVIII	3,9-Epoxy-11-keto-etiocholanic acid	345				247		

TABLE VII

SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF 100 μG. OF STEROID IN 3.0 ML. OF CONCENTRATED SULPHURIC ACID C.P.

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-2}$)											
	345–343	337–335	330	327–320	315	277–270	248–244	240–238	235	229	225–222	217–213
Estrone		2.60	2.52								1.84	
Estriol		1.02	1.02								1.92	
Estradiol-17β						(1.08)			4.59			
Progesterone	1.96					No absorption						
Testosterone		3.48									2.27	2.17
Desoxycorticosterone	2.05					1.03				0.58		
Dehydroepiandrosterone				1.16			1.98					
Androsterone				1.30			0.35	0.35			(0.28)	
Epiandrosterone				1.24			0.52					0.30)
Cholesterol				1.03				0.34				
Cholestanol-3				0.76	0.83			0.33				

hydroxyl or ketone; it is further evident that the absorption by the C_{11} group is dependent upon the system at the C_3 position. The persistent maxima in the 35,000–35,700 cm.$^{-1}$ region in the presence of a ketone group conjugated to a double bond is not unlike that observed with the formic and phosphoric acids when protonation to form the oxonium ion seemed the most likely reaction. However, this band is evident if the double bond is absent, and with an α -OH group in a saturated A ring.

A series of measurements in concentrated sulphuric acid C.P. solution, of a group of steroids of widely different derivation, were carried out (see Table VII). The regularities noted in Table VI are lacking; no consistent relationships of absorption with chemical groups were evident. The three estrogens are not similar; the conjugated keto steroids absorb within the region 33,500 cm.$^{-1}$ to 34,500 cm.$^{-1}$ but this overlaps the estrogen region. Compounds possessing the C_3 hydroxyl group in a saturated A ring showed absorption bands in two regions, 24,000 to 24,800 cm.$^{-1}$ and 32,000 to 33,000 cm.$^{-1}$. Visible absorption occurs only in the presence of hydroxyl group.

This suggests that the C_{17} side chain in Zaffaroni's series is exerting a regulating effect on the C_{11} and C_3 positions, through induction or resonance effects (see Fig. 2).

A cross comparison of the effects in the concentrated acids may be made (see Fig. 3). The ultraviolet absorption associated with the conjugated ketone group, and with the phenolic group, has moved to lower wave numbers in concentrated sulphuric acid. Presumably a marked change in the equilibrium constants of the protonation reactions has occurred in the last solvent. The absorption at 22,200 cm.$^{-1}$ of protonated C_{17} ketone, in estrone, remains the same in both phosphoric and sulphuric acids. Presumably protonation of this group would occur more readily than protonation of phenolic hydroxyl, therefore the latter is unlikely to have occurred in the formic acid, Table II. The C_3 and the C_{17} hydroxyl groups absorb at 24,000–24,800 cm.$^{-1}$ and 23,500 cm.$^{-1}$ respectively, when carbonium ion formation predominates.

Concentrated Sulphuric Acid Subsequently Diluted

Changes in the absorption spectra of the steroids in concentrated sulphuric acid can be brought about by subsequent dilution of the acid solution, either by water or a miscible organic solvent. This effect, on a group of available steroids of differing structure and origin, was studied by Linford (17, 19, 20); water or ethyl alcohol was added in increasing proportions to the acid solutions.

In the ultraviolet region the changes in position and intensity of the bands were such that a sequence of bands of increasing wave number could be discerned if the steroids were arranged in order of carboxylic, alcoholic, ketonic, and phenolic types (19). In the region below 28,000 cm.$^{-1}$ progressive dilution revealed a relationship between specific chemical groups and the position and intensity of absorption bands. In addition to this, each absorption band was displaced from its "primary" position to a "secondary" position of longer

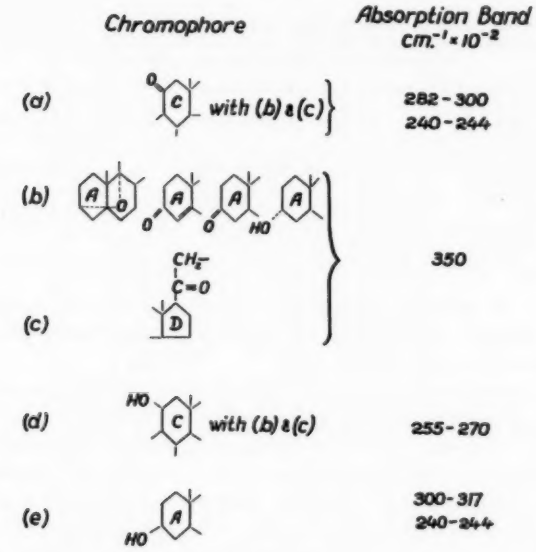


FIG. 2. A summary of the relationships between the absorption bands and the chromophores in adrenocortical steroids.

Chromophore	Resonant System	Reagent	Absorption Bands cm.⁻¹ × 10⁻²
(a)		90% H. COOH	385 - 388
		98% H. COOH	385 - 388
		H_3PO_4 85%	375 - 380
		C_6H_5. COOH	360 - 370
		H_2SO_4	335 - 345
(b)		H_3PO_4 85%	215 with (a) 220 with (c)
		98% H. COOH	240, 190 } with (c)
		H_2SO_4	222-5 with (a) 235 with (c)
(c)		H_2SO_4	335-337 330 222-5 with (a)
		90% H. COOH	380 - 383
		98% H. COOH	380 - 383
		H_3PO_4 85%	363

Chromophore	Resonant System	Reagent	Absorption Bands cm.⁻¹ × 10⁻²
(d)		H_3PO_4 85%	254-262, 242-248 } with (e)
		H_2SO_4	320-327 244-248 236-240
(e)		H_3PO_4 85%	220, 180, 168 } with (c)
		H_2SO_4	222-5 with (c)
		$SbCl_5$	338, 318, 247, 184-7 } with (d)
(f)		$C.Cl_3.COOH$	370 175 160

FIG. 3. A summary of the absorption bands of steroids in concentrated acids, and in salt solutions, in relation to the chromophores and their hypothetical states in these solvents.

TABLE VIII

ASSOCIATION OF STEROID GROUPS WITH ABSORPTION MAXIMA, WHICH APPEAR ON PROGRESSIVE DILUTION WITH ALCOHOL OF SOLUTIONS OF STEROIDS IN CONCENTRATED SULPHURIC ACID (19)

Chemical groups	Primary maxima (cm.$^{-1}$)	Secondary maxima (cm.$^{-1}$)
Alcoholic C_3 -OH	25,500–24,000	21,000–20,500
Alcoholic C_{17} -OH	23,000	20,500
Phenolic C_3 -OH	22,000	19,700
Ketone $\rangle C{=}O$	22,000	16,800

wave lengths, as dilution progressed. The attempt to summarize these associations, though they might be dependent upon interactions between groups, is shown in Table VIII.

The bands produced at one particular dilution, namely by addition of an equal volume of methanol in the cold, are shown in Table IX. Interactions between groups are evident: the maxima at 16,800 cm.$^{-1}$ are associated with the ketone group, but become intense in the presence of a double bond not necessarily in the conjugated position; in the absence of an hydroxyl group the band is absent as with progesterone. The estrogens possess two groups associated with characteristic absorption but one appears to predominate; estradiol-17β did not show the phenolic -OH band in diluted acid; estrone did not show the ketone band; estriol did not show the C_{17} -OH band.

Dirscherl and Breuer (8) have published a study of the effect of diluting concentrated sulphuric acid solutions of the steroids; they compared water and alcohol diluents. The wave number positions of the absorption maxima are listed in Table X. The change from primary to secondary maxima of the estrogens is shown to be dependent upon the nature of the diluent. Compare with Table VIII. Absorption at 16,700 cm.$^{-1}$ occurs with the steroids possessing unsaturation in rings A or B, and 3 keto or 3β -OH groups; those steroids having hydroxyl at the C_{17} position or side chain show this band in water–acid and alcohol–acid; those having no hydroxyl at C_{17} or side chain still show the 16,700 cm.$^{-1}$ band in alcohol–acid only.

Allen, Hayward, and Pinto (1) measured the visible absorption of a number of sterols in acid solution. These authors used 2.0 ml. of a reagent made up of one volume of 90% ethanol added to four volumes of concentrated C.P. sulphuric acid. They heated the solution for 12 minutes at 55° C. and added 3.0 ml. of 95% ethanol. Their results are listed in Table XI; the 19,700 cm.$^{-1}$ maximum of estrone is characteristic of the phenolic -C_3 hydroxyl group; the four neutral steroids possessing an hydroxyl group at the -C_3 position in the saturated A ring show an absorption maximum at 25,600 cm.$^{-1}$; the unsaturated keto sterols and androstenediol absorb at 16,700 cm.$^{-1}$.

A further example of the regularities in the low wave number absorption spectra of the estrogens is shown by the work of Cohen and Bates (6). These authors dissolved estrogens in concentrated sulphuric acid, added a series of

TABLE IX

SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF 100 μG. OF STEROID IN 3.0 ML. 1 : 1 : : SULPHURIC ACID: METHANOL*

Steroid	Optical densities at wave numbers ($cm.^{-1} \times 10^{-2}$)										
	280	275–270	260–258	248–242	240–238	230	221	210	207–200	198	168–166
Estrone	0.40						10.33			8.53	
Estriol							0.889			0.717	
Estradiol-17β	0.72					3.645			1.605		
Progesterone	No absorption										
Testosterone		1.148	1.104						1.267		5.95
Desoxycorticosterone		0.705									2.28
Dehydroepiandrosterone			0.909	1.141					(0.545)		1.637
Androsterone			0.468	0.472				0.363			
Epiandrosterone			0.482	0.502					0.231		(0.079)
Cholesterol					0.427			0.356	0.319		
Cholestanol-3					0.451			0.333	(0.308)		
Chemical groups	Hydroxyl or ketone at C_{11} or C_{17} side chain			Alcoholic C_3 -OH		C_{17} -OH	Phenolic C_3 -OH	Alcoholic -OH		Phenolic C_3 -OH	Ketone hydroxyl double bond

*Steroids in concentrated acid heated 1.0 hour at 52° C. Equal volume of methanol added in the cold.

TABLE X

RELATION BETWEEN STEROID CONSTITUTION AND POSITION ON ABSORPTION MAXIMA IN SOLUTION IN CONCENTRATED SULPHURIC ACID AFTER DILUTION WITH EQUAL VOLUME OF WATER (W), OR OF ALCOHOL (A) (8)

Steroid	Wave numbers ($cm.^{-1} \times 10^{-2}$)												
	W	A	W	A	W	W	A	W	A	A	A	W	A
Estrone					222					196			
Estradiol			232							196			
Dehydroepiandrosterone												167	167
Δ^5-Androstene-3β,17α-diol								204	208			167	167
Testosterone	260							206	208			167	167
11-Desoxycorticosterone	250					213	213					167	167
Progesterone								204	208				167
Δ^4-Androstene-3,17-dione	260							206	208				167
Δ^4-Cholesterone								204	208				167
Δ^5-Pregnene-3β,ol-20,one								204	208				167
Cholesterol	256							200			172		
Dihydrocholesterol				227				204			182		
Androsterone	250	244					213	200					
Pregnane-3α,20α-diol								206			185		

TABLE XI

SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF 100 μG. OF STEROL IN 10.0 ML. OF AN ALCOHOLIC SULPHURIC ACID SOLUTION (1)

Sterol	Optical densities at wave numbers ($cm.^{-1} \times 10^{-2}$)							
	310	256	213	211	208	197	183	167
Pregnanediol		0.46			0.40			
Dehydroepiandrosterone		0.14						0.71
Androsterone		1.28	0.66				0.42	
Androstenediol		0.17		0.26				0.40
Testosterone			1.20					0.76
Desoxycorticosterone	0.15				0.15			0.67
Estrone						0.23		

TABLE XII

SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF ESTROGEN AND ESTRONE IN CONCENTRATED SULPHURIC ACID SUBSEQUENTLY DILUTED BY THE METHOD OF COHEN AND BATES (5)

A. 100 μG. OF ESTROGEN IN CONCENTRATED SULPHURIC ACID DILUTED TO 10.0 ML.

Estrogen	Density of 1.0 cm. in 45% H_2SO_4 at wave numbers ($\times 10^{-2}$)		
	230	204	198
Estradiol-17β	0.348	0.495	0.482
Estrone			0.782
Estriol			0.691

B. A SERIES OF SOLUTIONS OF 100 μG. ESTRONE IN CONCENTRATED SULPHURIC ACID DILUTED TO 10.0 ML.

Conc. H_2SO_4, % by volume	Density 1.0 cm. at wave numbers ($\times 10^{-2}$)			
	221	220	218	198
91.5	1.34			
79.0		1.58		
58.0			1.58	
37.4			(0.280)	0.778
25.0			(0.202)	0.628
17.0			(0.140)	0.478

acid–water diluents, and heated the resulting solutions at 100° C. for 3 minutes. Densities calculated from their data are listed in Table XII. Estradiol-17β, under these conditions, shows both the absorption at 23,000 cm.$^{-1}$ associated with the C_{17} -OH group and the absorption at 19,800 cm.$^{-1}$ characteristic of the phenolic C_3 -OH group.

In the case of the estrogens, the specific absorption due to the effect of the concentrated acid on a given group is evidently masked by the acid environment and complicated by the interaction of one group upon another. Dilution of the environment at first produces a gradual change of absorption to slightly lower wave numbers, to reveal specific bands (the primary maxima) associated with each group. This process is probably the result of a change in equilibrium constant by weakening the acid environment. This is followed by a stepwise change to an appreciably lower wave number. These effects are exemplified in Table XII (B). The change from 22,100 to 21,800 cm.$^{-1}$ is continuous with little change in intensity; the absorption at 21,800 then decreases rapidly in intensity and a new maximum appears at 19,800 cm.$^{-1}$.

Diluted Sulphuric Acid

The use of acid diluted prior to reaction with the steroids might be expected to show more selective behavior with regard to dehydration, carbonium and oxonium ion formation, and, in addition, show environmental effects owing to interaction of the diluent with the acid.

TABLE XIII

OPTICAL DENSITIES AND SPECTRAL POSITIONS OF THE ABSORPTION MAXIMA OF 100 μG. OF ESTROGENS IN 10 ML. OF DILUTED SULPHURIC ACIDS (29)

Conc. H_2SO_4 (% by volume)	Optical densities at wave numbers ($cm.^{-1} \times 10^{-3}$)		
	235–230	223–216	198–195
Estradiol-17β			
100	0.798	(0.398)	
90	0.860	0.657	
75		0.996	
60		0.782	0.459
50		0.521	0.375
45		0.298	0.169
Estradiol-17α			
100	0.250	0.240	
90	(0.320)	0.463	
75		0.942	
60		1.16	0.320
45		0.494	0.927
30			0.418
Estrone			
100		0.668	
90		1.31	
75		1.37	
60		0.816	0.519
45		0.080	0.120

Umberger and Curtis (29) measured the visible absorption of estrogens dissolved in a series of sulphuric acid solutions by heating at 100° C. for 12 minutes. Their results are shown in Table XIII.

Three distinct regions of absorption are apparent. Absorption in the 23,000 $cm.^{-1}$ region is associated with the C_{17} hydroxyl group; absorption in the 22,000 $cm.^{-1}$ and 19,800 $cm.^{-1}$ regions is associated with the phenolic C_3 hydroxyl group. With progressive dilution of the sulphuric acid reagent, the absorption characteristic of the C_{17} -OH disappears and the remaining maxima move to lower wave numbers (17).

Bates and Cohen (3) used 90% (by volume) sulphuric acid as a solvent for estrogens and measured the visible fluorescence of the solution after further dilution. Their method was to add 0.2 ml. of an alcoholic solution of the steroid to 1.0 ml. of the 90% acid; this mixture was heated at 80° C. for 10 minutes, cooled, and diluted with 6.0 ml. of 65% (by volume) sulphuric acid. Braunsberg (5) measured the visible absorption of the estrogens in the Bates and Cohen solution; single absorption maxima at 22,000 $cm.^{-1}$ were shown by estradiol-17β, estrone, and estriol.

Using the method of Bates and Cohen, the absorption of a representative number of steroids, throughout the region 46,000 to 14,000 $cm.^{-1}$, was

TABLE XIV

SPECTRAL POSITIONS AND OPTICAL DENSITIES (1.00 CM.) OF THE ABSORPTION MAXIMA OF 100 μG. OF STEROID IN 7.2 ML. OF 67% SULPHURIC ACID IN WATER

Steroid	Optical densities at wave numbers (cm.$^{-1}$ × 10^{-2})							
	430	380	367	360	348	335	330	325
Estrone		0.362	0.349			0.376		0.476
Estriol		0.242	0.242			0.340		0.315
Estradiol-17β	0.676					0.380		
Progesterone					0.540			
Testosterone						1.65		
Desoxycorticosterone	1.05			0.955				
Dehydroepiandrosterone							0.414	
Androsterone								0.385
Epiandrosterone							0.339	
Cholesterol							0.536	0.546

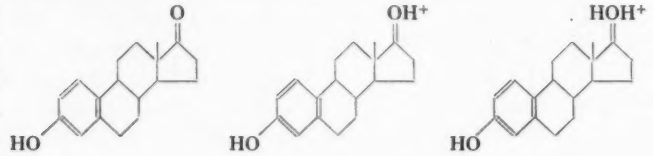
	Optical densities at wave numbers (cm.$^{-1}$ × 10^{-2})				
	275	264	250–245	220	167
Estrone				1.57	
Estriol				0.619	
Estradiol-17β	0.431			0.985	
Progesterone	No color				
Testosterone	No color				
Desoxycorticosterone	0.412			0.205	0.195
Dehydroepiandrosterone			0.227		0.462
Androsterone	0.249	0.207	0.098		
Epiandrosterone		0.475			
Cholesterol	No color				

measured in this laboratory. The results are shown in Table XIV. This reagent is more selective, the band systems are simplified, and the number of steroids showing color is reduced. The estrogens possess a single common band in the visible region and similar absorption bands throughout the ultraviolet.

An example of the selective action of diluted sulphuric acid is the specific colorimetric test for dehydroepiandrosterone by Munson *et al.* (23). This sterol produces an absorption band at 15,500 cm.$^{-1}$ when treated first with 16 *N* sulphuric acid, then with a dilute solution of furfural. The authors conclude that color production depends upon an -OH group or double bond in ring A, unsaturation in ring B, and oxidation anywhere in the molecule. These are the same requirements as for production of the intense 16,700 cm.$^{-1}$ absorption when a steroid in concentrated sulphuric acid is diluted with methanol; in both these cases, the weaker sulphuric acid may cause dehydration, and possibly form a carbonium ion, at the C_3 position only in the presence of the double bond. The interrelationship of these color reactions is shown by the occurrence of a fluorescence band at 15,500 cm.$^{-1}$ when dehydroepiandrosterone in sulphuric–methanol is irradiated within the 16,700 cm.$^{-1}$ band (20).

The absorption maxima of the estrogens, in concentrated and dilute acid solutions, may be explained in terms of protonation and carbonium ion formation.

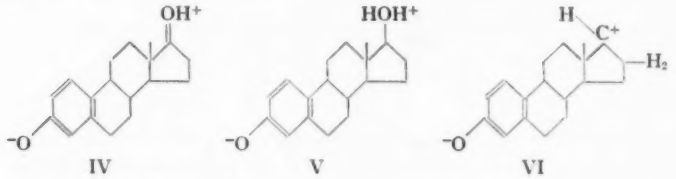
The absorption spectra of estriol and estrone are similar when these compounds are dissolved in concentrated sulphuric acid, when this acid solution is subsequently diluted with water or alcohol, and when diluted sulphuric acid is used as solvent. Both estrogens show strong maxima at 22,000 cm.$^{-1}$ in concentrated acid, which is replaced by maxima in the 19,800 cm.$^{-1}$ region in more dilute acid solutions (29). As estriol readily loses water, these absorption bands are probably associated with the estrone structure I. Estradiol-17β produces the same band system as estrone and estriol only when dilute acid is used as the solvent, i.e., when dehydration does not occur. Absorption at 22,000 cm.$^{-1}$ is, therefore, postulated to occur when estrone and estriol are in state II, and estradiol-17β is in state III. Protonation of the phenolic oxygen may also occur, but it is reasonable to assume that the neutral groups would do so more readily.



I II III

With increasing dilution of the acid solvent, the absorption intensity at 22,000 cm.$^{-1}$ rises initially. This is followed by a stepwise shift of the absorption maximum from 22,000 cm.$^{-1}$ to 19,800 cm.$^{-1}$, presumably owing to

ionization of the phenolic hydrogen, to form states IV and V. Continued dilution of the solvent decreases the optical density rapidly owing to breakup of the complex at the C_{17} position.



IV V VI

In concentrated acid estradiol-17β absorbs at 23,000 cm.$^{-1}$. Further dilution produces a stepwise shift of the maximum to 20,700 cm.$^{-1}$. In concentrated acid water is lost to form the Δ^{16} compound; in the presence of $[H]^+$ ions, carbonium ion is formed. With increased dilution, ionization of the phenolic hydrogen occurs to some extent; this absorbing state is represented by VI.

The ideal solution for maximum absorption and fluorescence would contain one charged state. In the cases where several states exist in equilibrium with the solvent, a number of individual fluorescence bands may be produced by the use of incident exciting light of different wave lengths (17).

A marked rise in the absorption intensity at 16,700 cm.$^{-1}$ of those steroids possessing the keto group and unsaturation in the A or B rings occurs when the acid solution is diluted. This band has been represented as a secondary to a weak band at 22,000 cm.$^{-1}$ in Table VIII. Taking dehydroepiandrosterone as an example, the effect of different diluents was studied. The increase in absorption at the lower wave length with water, methanol, acetic acid, and ethyl acetate diluents was similar; acetone altered the absorption very little in comparison. The effect of the keto group at the C_{17} position also exerted a marked influence on the absorption at 24,000 cm.$^{-1}$ associated with the C_3 hydroxyl group. Fig. 4 shows a reciprocal relation between the C_3 and C_{17} color reactions; an isosbestic point is indicated in the region of 20,000 cm.$^{-1}$. This type of interaction has been noted earlier. Fig. 4, furthermore, shows an increase in absorption intensity at 16,700 cm.$^{-1}$ with an increasing tendency of the diluents acetone, acetic acid, methanol, to accept a proton from the sulphuric acid. The color change accompanying the dilution is, therefore, governed by the extent of interaction of the added diluent with the concentrated acid; it acts by production of ions in the environment, not by simple dilution.

These absorption results parallel those obtained with $SbCl_3$; the C_3 -OH group is required in conjunction with the ketone group and the same sharp maximum at 16,700 cm.$^{-1}$ is produced. In this case, however, unsaturation in the molecule is necessary to produce high intensity.

Two possible effects may be considered. Dilution will alter the equilibrium constants of protonation, and between the carbonium ion and the ions in its

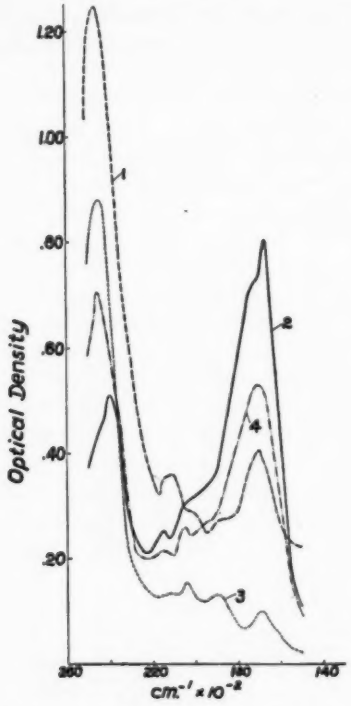
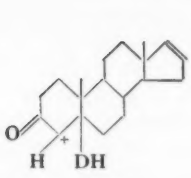


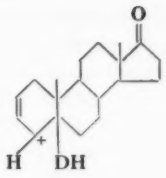
FIG. 4. Absorption of dehydroepiandrosterone, initially dissolved in concentrated sulphuric acid and heated 1.0 hour at 52° C., then added to an equal volume of: (1) acetone; (2) methanol; (3) sulphuric acid; (4) acetic acid. Final concentration 17 μg. per ml.

environment. Each of these is a resonating system; if this result of dilution then allowed coupling of these systems to extend the resonating system across the nucleus, the shift of absorption to longer wave lengths would be accounted for. However, this intercoupling has been used to explain a number of the absorption spectra in the concentrated acids (Fig. 3).

Moreover, such an explanation does not readily account for the specific effects of the particular ions produced, and particularly does not account for the further lowering of the absorption wave numbers in the case of furfural. The latter effect appears to be due to the incorporation of its own unsaturated system into the resonating system. Furthermore, Tables X and XI show this 16,700 cm.$^{-1}$ band to be present when the ketone group at the C_3 position is replaced by β hydroxyl. The C_{17} group also influences the environmental conditions under which the band appears, but the essential group appears to be the double bond. We postulate that the ions formed in the solvent are linked to one of the unsaturated carbons. The absorbing systems may be represented as VII and VIII, where D = R.OH, R.CHO, etc.



VII



VIII

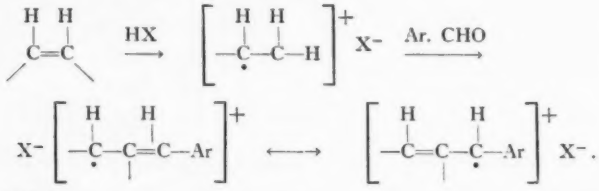
Woker and Antener (31) have tabulated the colors of series of constitutionally related steroids when an alcoholic solution is added to an equal volume of concentrated sulphuric acid. The results are qualitatively in agreement with those described earlier; no color appeared with cholestanone, progesterone, and androstane-3,17-dione. The appearance of color in the presence of -OH groups depended upon the reactivity of the group. Of the four isomeric androstanediols the color intensity was a maximum if both hydroxyl groups were in the *cis* configuration and a minimum if both were *trans*, with reference to the C_5 hydrogen atom. Of the two isomeric androstane-3-one,17-ol steroids, only the one having the hydroxyl group *cis* with reference to the C_5 hydrogen atom showed color. In Fieser's notation, the α configuration of the C_{17} -OH group is the more reactive.

However, these authors state that adding a trace of furfural produces color, both with the β compounds and with the keto steroids possessing no hydroxyl group. The color is weak.

Color reactions of a similar nature are those of Lieberman and Salkowski, more recently elaborated by Whitby (30). The sterol in carbon tetrachloride or chloroform solution is shaken with sulphuric acid; the liquids being immiscible, the product of reaction with the acid is redissolved in the organic layer. Colored products are formed if the sterol contains a double bond as in cholesterol. Whitby showed that dehydration of the sterol first occurred to produce a $\Delta^{3,5}$-cholestadiene type of compound. The colored product resulted from a further interaction with formalin, acetic anhydride, benzoyl chloride, or sulphuric acid; the latter is relatively inefficient, therefore addition of small amounts of the other materials appears to increase the sensitivity of the color reagent greatly. The step producing the color complex is retarded by traces of water, therefore, acetic anhydride or phosphorous pentoxide is necessary to dehydrate the medium.

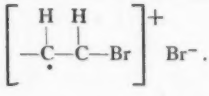
Miescher (14, 22) makes use of presumably milder dehydration conditions with sulphuric acid in acetic acid to distinguish between *cis* and *trans* C_{17} -OH groups. Using the weaker acid, the addition of bromine, acetic anhydride, aldehyde, or chromic acid is required to produce the colored complex. A trace of these latter substances is often more effective than a high concentration of mineral acid. The author therefore suggests the sequence of reactions to be: (*a*) loss of -OH group to form a double bond; (*b*) carbonium ion formation at the double bond; (*c*) conjugation of the carbonium ion with an unsaturated grouping. This produces a halochromic salt, the positive ion of which is a resonating system.

Assuming that the $\Delta^{16,17}$ double bond is initially formed, as suggested by Boscott, the sequence of reactions would be:



with loss of H_2O.

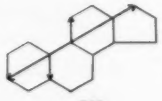
In a similar manner the carbonium ion could react with halogen to form:



In this type of reaction, the color is produced at one grouping, the double bond. Protonation presumably does not occur and interaction of groups across the steroid nucleus is not a factor. However, as discussed above, the carbonium ion could be formed by addition of a protonated form of the color producing reagent to the double bond.

Conclusions

A unified explanation of the color reactions of steroids may be made by assuming the formation of charged structures when oxygen-containing groups are present at the C_3, C_{11}, and C_{17} positions in the nucleus. Acid reagents form carbonium and oxonium ions, metallic salts form molecular compounds. Hypothetical formulas for these charged structures are shown in Fig. 3. The effect of diluents, and of small amounts of such oxygen-containing substances as aldehydes, is to extend the charged structures. In many cases the absorption of light depends upon the interaction of the charge-carrying groups at the C_3, C_{11}, and C_{17} positions; unsaturation in the A or B rings acts as a modifier of the C_3 reactivity. The steroid molecule appears to possess an axis (IX).



IX

The biological activity of steroids is related to the presence of specific combinations of certain keto, hydroxyl, and unsaturated groupings. The results indicate that the steroid nucleus, through induction or resonance effects, permits an influence of such groups upon one another, particularly when they are located at the C_3, C_{11}, and C_{17} positions. The color tests suggest that the degree of interrelation is governed to a great extent by the environment.

By the use of color reagents, steroids have been chemically activated to yield products having antirachitic properties (9). The active form of the steroid in a physiological environment may be a charged structure.

Variations in the intensities of color reactions cause difficulties in their application to assay procedures. As the charged structures exist in equilibrium with the ions in the environment, slight changes in the latter would be expected to have an appreciable effect on the concentration of the former and on the absorption intensity. The association of color with the concentration of ions in the solvent also explains the frequent failure of Beer's Law at low steroid concentrations.

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Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

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